

Reductionists lay claim to the mind

There are deep disagreements about how science should deal with the problem of consciousness. But neuroscientists should soon be in a position to set the agenda. The result is likely to be rapid progress.

ALTHOUGH the object of their study is also the organ that produces consciousness, neurobiologists are often reticent on the topic following their failure so far to reach any agreement about how (if at all) this phenomenon should be studied. But they have recently begun to raise their heads above the parapet, to join equally rash physicists, philosophers, psychologists and computer scientists. This community is united in accepting that consciousness has something to do with the brain (and that neurobiology might therefore be relevant), but in little else.

Perhaps the most basic question is whether existing physical principles are sufficient to explain the brain. The boldest attempt to 'rescue' consciousness from neurobiological reductionism comes famously from Roger Penrose. He argues that the brain performs feats of understanding that cannot be computed and so cannot be implemented by any system based on known physical laws. Instead, he believes, explaining the brain will require a new theory linking events at the quantum level with those of the macroscopic world. Adherents to this view invoke key bits of cellular infrastructure — microtubules — as the most likely substrate for coherent quantum-level phenomena, but most neurobiologists are unimpressed. It remains to be demonstrated that quantal processes might show unusual manifestations in microtubules, and it is difficult to see how they could influence or be influenced by neurotransmitter release or electrical depolarization of neuronal membranes, events with undoubted relevance to mental processes.

Most importantly, there is no direct evidence that current physical concepts are inadequate to explain the brain, or that a quantum-level theory would be any better. Penrose believes that neurobiologists will indeed encounter phenomena that cannot be accounted for by known principles, perhaps even in relatively simple nervous systems. But, unless this happens, neurobiologists might best treat his argument as an obstacle to be side-stepped rather than as a source of illumination. That certainly seemed to be the majority response to his views at a multidisciplinary meeting on this topic ("Towards a science of consciousness") last month in Tucson, Arizona, which provided a snapshot of current debates.

If the brain can be entirely explained in terms of known physical processes, then perhaps consciousness has no independent causal effects. How then was it favoured by natural selection? Perhaps it wasn't. Consciousness may simply be an epiphenomenon of certain arrangements of matter, or indeed of all matter: as one speaker at the conference remarked, perhaps even an atom feels a spasm of relief as an electron falls to a lower orbital. Complex brains would presumably generate more complex forms of consciousness as a by-product of their more sophisticated computations. But this is not very helpful in explaining why (say) the colour red feels the way it does, or in deducing from the neurobiology of echolocation how it feels to be a bat, or in deciding whether it is unethical to unplug a computer. David Chalmers (whose book *The Conscious Mind* is reviewed on page 123) refers to this class of questions as "the hard problem"; unlike the (relatively) easy problem of explaining how the brain generates behaviour, the experiential aspects of our brain states seem to defy any conceivable explanation in terms of known concepts.

But not everyone accepts this 'hard/soft' distinction; for one thing, it tends to marginalize neuroscience by implying that it can

never hope to illuminate the 'hard' problem. Being marginalized, however, does have the advantage of leaving neuroscientists free to tackle the 'easy' problems untroubled by philosophical angst. While the full experiential aspect of consciousness may be impossible to define or measure, an 'easy' definition is both feasible and useful: to be conscious of something is to have a flexible neuronal representation that can be used to drive many different behavioural outputs. In an experimental situation, this can be an arbitrarily specified button press or (for humans) a statement that one is conscious. The subject's testimony can then be correlated with the accompanying neural activity, either by functional brain imaging or (in monkeys trained to report their perceptions) by recording the activities of individual neurons.

So far, most such studies have employed visual stimuli, but other approaches, along with conditions such as sleep, anaesthesia, and perhaps even trance, offer further opportunities to search for neural correlates of conscious states. The underlying assumption is that systematic correlations are there to be found, and that conscious states correspond not to some loosely defined global state of the entire brain, but much more precisely to the activities of specific populations of neurons.

Whatever the nature of these patterns of activity, credit is due to Daniel Dennett for warning against the "Cartesian Theatre" fallacy that perceptual representations converge on a single hypothetical "perceiver". Our current knowledge of the brain gives no support to such a view; visual representations, for instance, are distributed across many brain regions. Similarly, Dennett doubts the existence of any "central meander" that decides what we mean to say and do. Instead, he envisages that multiple neuronal representations are constantly competing for control of both perception and action. And to those who reply that "it just doesn't feel like that", he rightly warns against the assumption that a few minutes of introspection are sufficient to test a theory of brain function.

Those who still think that their intuitions are a reliable guide to how their minds work should reflect on the condition known as anosognosia. This rare syndrome arises after damage to the right parietal lobe, and leaves patients not only paralysed on the left side of their bodies but also unable to acknowledge the fact. When asked to perform tasks with the paralysed hand, they confabulate, and many will also deny the paralysis of a fellow patient. And as if this were not bizarre enough, the false belief (though not the paralysis) can be temporarily corrected by flushing cold water into the left ear. There is reason to believe that anosognosics have suffered damage to a "belief mechanism" that normally updates our world-view in the face of new information; it should now be possible to identify the brain regions involved.

As neuroscience sheds light on perception, memory, emotion, decision-making and belief, it will become increasingly difficult to deny its central importance in understanding consciousness. Many other approaches will come to seem correspondingly irrelevant. But whether neuroscientists will be able to make their discoveries 'feel' like an explanation for what goes on inside our heads, whether we will ever be able to reconcile their findings with our intuitions about our own mental lives, seems less certain. To meet that challenge, neuroscience may yet be grateful for the philosophers' help. □



Antarctica research essential for continued influence, says panel

Washington. The United States must continue its research activities in Antarctica if it wants to maintain its political influence in the region, according to a report to Congress from the National Science and Technology Council (NSTC), the body that coordinates science policy in President Bill Clinton's administration.

The report — requested by senators who have challenged the value of the \$200 million spent each year on Antarctic activities by the National Science Foundation (NSF) — applauds the quality of the science being done there. But it emphasizes that the chief justification for the programme, including a year-round presence at the South Pole itself, is to secure US influence in the administration of the Antarctic Treaty.

The 1959 treaty prohibits military activity on or near the continent and imposes a moratorium on the pursuit of territorial claims, including conflicting ones between Argentina, Chile and the United Kingdom. In a memorandum attached to the NSTC report, the US State Department argues that abandoning the South Pole station would create a vacuum, whose likely result would be "a scramble to occupy the site, to the detriment of our position as well as to the stability of the Treaty system".

The memorandum argues that the programme earns the United States "a decisive role" in Antarctica under the treaty, which divides influence among the signatory nations on the basis of their actual activity on the continent.

But the NSTC falls short of recommending the construction of a new South Pole station to replace the current, rather dilapidated one until "further cost-benefit analyses" have been done. The highest priority, it says, is a \$25-million emergency repair programme, which is part of the NSF's 1997 budget proposal.

The Antarctic science programme provides vital input in the study of global systems, including continental drift, climate change and ocean circulation. The continent also serves as a unique natural laboratory because of its isolation, making it a useful platform for astronomers. And the geography itself is of great interest to oceanographers and geophysicists. But research is expensive, with logistics consuming two-thirds of NSF's investment.

The panel report says that the main McMurdo station and its two outposts — the Palmer station and the Amundsen-Scott

station at the South Pole — should all stay open. But it also says that a new panel of logistics experts should be brought together by NSF to establish how this can be done if

Flying the flag: but no new money for rebuilding.

funding is frozen.

Neal Sullivan, head of polar programmes at the NSF, says that the report's endorsement is "critically important" to the future of the programme. But he says that he is "disappointed that we do not have the green light" to rebuild the South Pole station.

"That is the next step, and that is what we need to work towards."

Gerald Garvey, of the White House Office of Science and Technology Policy, one of the authors of the report, says that the South Pole station still has "ten or fifteen years" of useful life left. But Sullivan says that with a new station taking four years to plan and eight to build, a start must be made quickly.

David Millar/SPL

The report was requested by the Senate Appropriations Committee at the suggestion of Ted Stevens (Republican, Alaska), who has a long-standing (and understandable) concern that most of NSF's polar programmes money goes to the pole which is far from his home state.

Administration officials hope that the report will at least help them make the case in Congress for the \$25 million of urgent repairs to the South Pole station. Last year, Congress cut \$20 million from the administration's request for polar programmes.

Colin Macilwain

Scrutiny delays Brent Spar report

London. Delays in the publication of a report written by a group of scientists at the invitation of the British government to evaluate the environmental implications of deep sea disposal of the Brent Spar, a disused oil storage buoy owned by the Shell oil company, has fuelled speculation that its conclusions are being viewed less than favourably by the government.

The inquiry was set up at the government's request by John Krebs, chief executive of the Natural Environment Research Council (NERC), shortly after Shell's decision to bow to public pressure and abandon plans to sink the buoy in the mid-Atlantic (see *Nature* **380**, 13; 1996). Its report was submitted to Tim Eggar, minister of energy at the Department of Trade and Industry (DTI), on 3 April, and had been expected to be launched at a press conference on 24 April.

No official reasons have been given for the delay. But some sources say that Eggar — who strongly favours deep sea disposal of the buoy — is unhappy with the report's conclusions. "He expected the scientists to endorse dumping the Brent Spar in the sea," says one individual who has seen the report. "But the scientists did not say that."

A spokesman for the department

acknowledges that the report has been seen by the minister, but refuses to say when it will be released. Helen Wallace, a senior scientist with Greenpeace, says the report should be published without delay. "Would they be so slow to publish a report that said exactly what they wanted?" she asks.

The group of "independent expert scientists" was set up to review the scientific, engineering and environmental aspects of deep sea disposal compared to other disposal options. But it did not have time to evaluate other options, and concentrated instead on the impact of deep-sea disposal.

One of the scientists, who did not wish to be named, confirmed that the report did not endorse deep-sea disposal as the 'best environmental option', but said that this was because such a recommendation had not been requested.

A difference of opinion is also believed to have emerged between the DTI and NERC over who should pay the costs of printing the report, and how many should be printed. The DTI is believed to favour restricted circulation of the report to a limited number of selected individuals, while NERC is said to be pushing for wider dissemination. **Ehsan Masood**

Synchrotron bids seek ways to raise money

Paris & London. Britain's research councils are said to be close to giving their formal approval to a £9-million (US\$13.5 million) upgrade to the 2-GeV Synchrotron Radiation Source (SRS) at Daresbury in Cheshire.

But the difficulties in securing funds for the upgrade suggest that finding the £100 million needed to build the SRS's proposed successor will be an uphill struggle. Indeed according to laboratory officials, it may need a novel funding approach, such as 'borrowing' construction money from the government — or even industry — which is then 'paid off' over the lifetime of the instrument.

Synchrotron radiation is emitted from beams of high-energy electrons or positrons when their path around a circular accelerator is bent by magnets. Modern machines maximize the production of such radiation using 'insertion devices' which force the particles to slalom about the linear part of their trajectory. This results in the intense beams of radiation which are widely used to probe the structure of matter.

The upgrade to SRS would greatly increase its capacity by adding two such insertion devices. But SRS itself will need to be replaced early next century, when it will have become obsolete. Indeed, the SRS is already outperformed by more modern facilities, such as the Advanced Light Source at Berkeley in the United States, the Electra facility in Trieste, Italy, and the Delta and Bessy II synchrotrons being built by Germany in Dortmund and Berlin.

British researchers already use the 6-GeV European Synchrotron Radiation Facility (ESRF), the world's most powerful synchrotron source, which was opened in Grenoble, France, in 1994. But ESRF, like the planned US Advanced Photon Source (see opposite) produces 'hard X-rays', which are used mainly to probe atomic structure.

Many researchers working on biological and other structures also require sources of 'softer X-rays' and ultraviolet radiation. To meet this need, British scientists are proposing the construction of a 'third generation' 3-GeV machine, known as Diamond, at a cost of about £100 million over five years.

The scientific case for Diamond was strongly endorsed last year by the council of the Central Laboratory of the Research Councils (CLRC). But little progress has been made since then, according to an official at the laboratory.

The delays expose what many feel to be the lack of an adequate financial mechanism for supporting big national science facilities in the United Kingdom, particularly after the reorganization of the research councils in the government's white paper of 1993.

As science budgets are squeezed around the world, funding agencies are facing increasing difficulties in raising the money needed for new, large research facilities. The dilemma is particularly acute where these facilities must compete for support against research programmes. On this and the following two pages, we report on particular aspects of this issue that are being faced in Europe, Australia and the United States.

Following, in particular, the splitting up of the former Science and Engineering Research Council (SERC), no individual council has sufficient funds to pay for such projects alone. Conversely, while the CLRC is now responsible for managing large facilities for its research council 'customers', it has no budget of its own for new facilities.

A further complication is the fact that the general squeeze on 'operating costs' in the UK science budget leaves little room for major investment in new equipment (see page 102), hence the idea of developing a new funding mechanism that allows separate procedures for large capital investments and running costs within the science budget.

At the same time, even if the research councils were to agree to finance Diamond, the approval process may be too slow for a decision to be reached within the next 18 months. This timescale needs to be met, according to Ron Newport, the director of the Daresbury Laboratory, if Diamond is to be built before the existing SRS becomes obsolete early next century.

Meanwhile, France and Switzerland have

DIAMOND: an expensive sparkle in the eyes of Daresbury's synchrotron planners.

Daresbury Laboratories

Australian astronomers are rebuffed in move to apply for

Sydney. Prolonged uncertainty among Australian astronomers has ended with news that the new government has decided not to support their bid for Australia to become the first non-European member of the European Southern Observatory (ESO).

Various scientific groups have already protested against the decision, which has shortened the honeymoon period for Peter McGauran, the new minister of science and technology. But McGauran says the proposed ESO subscription fee of \$A30 million (US\$23.7 million) is too expensive for a government planning cuts in public expenditure of \$A8 billion over the next two years.

The previous Labor government was first approached by a consortium of university and government astronomers two years ago for funding for ESO membership under the Major National Research Facilities scheme, worth \$A62 million over six years.

Australian astronomers had been invited to apply for partnership in the world's largest optical/infrared telescope (the Very

Large Telescope or VLT) being built at Paranal in Chile, partly to contribute their high level of expertise in optical interferometry. The subscription was to have been spread over several years, and the proposal had gained the unanimous and enthusiastic support of a peer review committee. It was ranked as the top project by government committees last year, which proposed support of \$A28 million over six years.

This support was overturned at cabinet level by the Labor government before its release of an Innovation Statement last December (see *Nature* 378, 653; 1995). But after protests from astronomers, led by Jeremy Mould, Director of the Mt Stromlo and Siding Spring Observatories of the Australian National University, the then Minister for Science, Peter Cook, reversed the decision and announced, during the election campaign, that ESO would be a top priority.

Following Labor's election defeat, Mould says that he put the astronomers' case personally to McGauran on 11 April,

including an estimate of "tens of millions of dollars' worth of contracts" for Australian local industry to build components for the VLT. He came away believing it that it had been "a constructive meeting".

But a spokesman for McGauran said last week that "the offsets were few, and not adequate reason for \$A30 million of support". He said astronomy in Australia already benefits from \$A32 million in annual funding, and that the Australia Telescope had received \$A11 million for an upgrade in the Innovation Statement. "Astronomers cannot claim they have been hard done by," he says.

The shock decision came in a letter to Mould from McGauran last week. It prompted an immediate statement of "disappointment" by Sir Gustav Nossal, the president of the Academy of Science, only hours before hosting the academy's annual dinner at which McGauran was present. Although Mould said the letter did not rule out the possibility of membership of ESO being sought again "some years down the

UK council seeks £60 million 'pulse' to ease LHC burden

London. Britain's Particle Physics and Astronomy Research Council (PPARC) is seeking an extra 'pulse' of funding of about £10 million a year over 6 years to help to offset the costs of its contribution to the Large Hadron Collider (LHC) being built by the European Laboratory for Particle Physics (CERN) in Geneva, Switzerland.

The plan represents a novel approach to the financing of major research facilities, as PPARC officials say that they would, if necessary, consider accepting this 'pulse' as a loan from government, to be paid off over the lifetime of the new accelerator, expected to be about 20 years.

But it also coincides with widespread criticism in the particle physics community of the first step in PPARC's declared strategy for remedying the budgetary crisis facing their discipline, namely that it intends to seek a reduction in its contribution to CERN — a move whose likely failure, say physicists, could leave them even worse off than they are at present.

The difficulties facing PPARC stem from two factors. One is the general squeeze on the science budget, coinciding with demands from researchers for increasingly expensive equipment. The second is a relative increase in the size of the UK contribution to CERN, due to a combination of the strength of the British economy, and the fall in value of the pound against the Swiss franc (in which the subscription is paid).

The result is that, at current funding levels, the £70 million (US\$105 million) a year that Britain's contributes to CERN — and which will be used to help build the LHC — will leave the domestic particle physics budget, needed to pay for the preparation of experiments on the machine, starved of funds.

Concerned at the extent to which the CERN contribution now dominates its whole budget — and in particular its impact on astronomy projects — PPARC's council agreed last month to 'cap' total spending for particle physics, including the CERN subscription, at a level of about £95.5 million a year.

This proposal, combined with a statement of its intention to seek a reduction in the CERN contribution in order to be able to increase domestic spending on particle physics, was included in the council's 'business plan', which has since been submitted to the government.

The problem, as PPARC officials admit, is that other CERN members are unlikely to listen sympathetically to the request for a reduction, particularly as the UK government can no longer justify such a request on the grounds of economic difficulties (as

Germany, for example, was able to do in the period immediately after reunification).

It was with this in mind that Ken Pounds, the chief executive of PPARC, told the House of Commons Select Committee on Science and Technology last week of the new funding proposal, pointing to what he described as the "lumpy" nature of investment in major research facilities.

Pounds said that the total amount of money needed both to help build the LHC and to participate adequately in experiments "considerably exceeds any expectation from our current plan". One way around this, he said, "would be to secure an extra injection of money over a finite period of time; but we need help from government to do that."

He suggested a figure of about £10 million year over the six years 1998 (the first year of construction of LHC) to 2003 to get over this problem. Peter Williams, chairman of PPARC — and of the company Oxford Instruments — justified this as being similar to a request for capital investment by an industrial company, to be written off over the lifetime of a project. "We want an exceptional capital fund during the lifetime of LHC that will relieve the pressure on the current revenue account."

Williams also promised that the controversial decision at the last meeting to cap the total funding for particle physics — a decision that could lead to serious difficulties if the CERN subscription is not reduced and no extra money is forthcoming from government — would be "revisited" at the council's next meeting in June.

This announcement has dampened some of the criticism of the research council's strategy from the physics community, which last month led those attending a 'town meeting' of particle physicists to the verge of passing a motion of no confidence in Pounds and his staff (see *Nature* 380, 576; 1996).

Asked whether the cap would be retained if there is no reduction in the CERN subscription, Williams said that it would be "unrealistic to imagine that we would fix a cap now and stay with it forever".

Peter Kalmus, professor of physics at Queen Mary College, London, said that he was relieved that "there seems to be a bit of backtracking", even though there had been no commitment yet to a change of policy. But Roger Cashmore, professor of experimental physics at the University of Oxford, and one of three authors of a letter to *The Times* last week criticizing PPARC, says: "We are still going to keep the pressure on".

David Dickson

'Voucher' scheme will give costed access to facilities

London. The main research council sponsoring the physical sciences in British universities is introducing a system of 'virtual vouchers' to provide support for the use made by academic researchers of major research facilities.

In the past, such facilities — for example, large lasers or synchrotron X-ray facilities, located primarily at the Rutherford Laboratory outside Oxford and at the Daresbury Laboratory in Cheshire — were financed directly by the research councils. Researchers then put in bids for time on the machines, without having to take account of the costs involved.

In future, all those applying for grants from the Engineering and Physical Sciences Research Council (EPSRC), which supports £210 million (US\$317 million) of research a year, will include in their application a request for a certain amount of time on large equipment.

The value of this time will have been calculated on the basis of running costs obtained from the laboratory operating the facility, and will be included explicitly as a cost in the grant application. If the grant is awarded, this money will not go to the researchers but directly to the laboratory concerned.

"There has been a strong feeling [at the EPSRC] that we need to get our use of facilities in order," says Richard Brook, chief executive of the research council. "When we looked at how our 'cake' is divided up, it became clear that a substantial proportion of our funding goes towards these major facilities, but that this money was somewhat out of control, as it was not being evaluated against other demands [on the research budget]."

According to Brook, this new way of operating is consistent with the decision, taken in the government's white paper of May 1993, to 'spin off' the Rutherford and Daresbury laboratories to a separate organization. Both are now the responsibility of a body known as the Council for the Central Laboratory of the Research Councils (CLRC).

As far as the laboratories are concerned, contributions towards their planned operating budget for any one year from the EPSRC will be at the level of the use made of the facility by EPSRC grant recipients in the previous year. "The laboratories are happy with this new arrangement, and we are happy with it," says Brook, who claims that the new system will make the whole process of paying for the use of major research facilities more 'transparent'.

D.D.

Ceiling principle 'not needed' in DNA cases

Washington. DNA samples can be used for positive identification of crime suspects with greater certainty than has been assumed, according to a report released last week by the US National Academy of Sciences (NAS). As a result, the academy recommends that prosecutors should no longer use the so-called 'ceiling principle' to estimate the probability of a suspect being mistakenly identified by DNA evidence.

The 'ceiling principle' was recommended by an academy panel in 1992 as a means of estimating the chances of a mistaken identity in cases — quite common in the US legal system — where the suspect belongs to the same racial group as the perpetrator, but where information is scarce about the level of shared genetic information among members of that group.

But the new report, prepared by a panel chaired by James Crow of the University of Wisconsin at Madison, abandons the 'ceiling principle'. It argues that, with better genetic information now available about different racial groups, prosecutors should estimate the chances of mistaken identity by more direct and less conservative means.

Crow says that the main reason for the change is "the great abundance" of data now available about different genetic populations. The panel's report states that the technology for DNA profiling, and the methods for estimating frequencies and related statistics, "have progressed to the point where the reliability and validity of properly collected and analysed DNA data should not be in doubt".

The academy agreed to take a new look at the role of DNA in legal proceedings at the request of the Federal Bureau of Investigation (FBI) — a move that prompted some scientists to complain that the academy's neutrality was being compromised (see *Nature* 367, 101; 1996).

The FBI had asked for the new study because it thought that the application of the 'ceiling principle' in US courts was occasionally leading to wrongful and unnecessary

acquittals. The report was paid for by the National Institute of Justice — a sister agency of the FBI in the Department of Justice — and other government agencies.

According to David Kaye, a panel member and professor of law at Arizona State University, in typical cases where the 'ceiling principle' has been employed, it might suggest a possibility of an error of 1 in 10,000, whereas a direct calculation would suggest a chance of only one in a million. In some cases, defence lawyers have been able to convince juries that the former statistic constitutes cause for reasonable doubt in reaching verdict.

The academy panel also calls for laboratories analysing DNA samples to "adhere to high quality standards" and to "make every effort to be accredited for DNA work". It says that "whenever feasible, forensic samples should be divided into two or more parts", so that the second sample can be independently analysed.

Critics of the report expressed disappointment not so much with the abandon-

ment of the ceiling principle — which they had expected — but with what they said was the weakness of the recommended provisions for imposing quality requirements on DNA laboratories and allowing for duplicate testing.

"I'm unhappy with their unwillingness to demand quality" from the laboratories, says Richard Lewontin, a population geneticist at Harvard University in Cambridge, Massachusetts, who had been strongly critical of the terms of reference for the new study. He says the requirement to divide the sample is of no value to most US defendants in serious criminal cases, as their public defenders have no money to buy a second test.

Panel members said that questions about the reliability of the laboratories analysing DNA samples will be dealt with more fully by an independent Laboratory Advisory Board recently established by the FBI under the chairmanship of Joshua Lederberg, the Nobel prizewinner and former president of Rockefeller University in New York.

Colin Macilwain

Group plans boost to German biotech

Munich. A new technology advisory group, established by the German Chancellor, Helmut Kohl, to suggest ways of improving the commercial exploitation of biotechnology, set itself a tough deadline this week when it promised to produce its report by early autumn, rather than by December, as Kohl had requested.

The group is only the second *Technologie*rat to have been established since Kohl created the concept two years ago. He identified biotechnology as an important topic for his technology advisers because of awareness by German politicians that Germany is missing an opportunity to profit from its industrial potential.

Germany's problems with biotechnology stem mainly from a cultural aversion to genetic engineering (known as 'gene technology'), which has led to biotechnological research and development being hindered by strict and bureaucratic legislation, as well as to relative underfunding.

Jürgen Rüttgers, the research minister, has been addressing the problems. New research programmes in biotechnology, for example, including involvement in the Human Genome Programme, have been launched in the past year by his ministry, which has also set up a group to investigate the general and legislative environment for research. Rüttgers has also said that biotechnology will be protected from the new cuts imposed on the ministry as part of an emergency budget being debated in parliament.

According to Detlev Ganten, director of the Max Delbrück centre for Molecular Medicine in Berlin, and a member of the new *Technologie*rat, the group does not intend to come up with any dramatic proposals, but will analyse recognised problems in detail, propose priorities and make other recommendations. He sees its major role as providing a 'kick-start' to a process already primed to take off.

At a four-hour meeting on 6 May, the *Technologie*rat, which includes representatives from the academic, industrial and political worlds, set up three working groups. They will look at technology transfer and scientific progress, at the general and legislative environment for biotechnology and at public acceptance of biotechnology. According to Ganten, the mood of the group was uniformly positive. For the first time, he says, industrialists appeared ready to believe that politicians were genuinely committed to improving their situation.

The *Technologie*rat's recommendations are likely to have considerable influence. The report of the first such group, set up last year to look at multimedia and information technology, was completed last December, and many of its recommendations are already being put into practice. But changing public opinion is a forbidding task, and the government may also find it difficult to provide the increased funding which the *Technologie*rat is likely to recommend.

Alison Abbott



Partners fall out over supercomputer bid

San Diego. A split has appeared in the academic/industrial partnership that has operated the San Diego Supercomputer Center for a decade about competition for a new grant from the US National Science Foundation (NSF).

The top three scientific administrators at the centre, which is run jointly by the company General Atomics (GA) and the University of California at San Diego (UCSD), have already resigned, and more key staff are thought to be about to leave.

NSF officials are monitoring the unprecedented strife closely because of concern that the centre might lose more staff. If so, it may be unable to fulfil federal contract obligations for the facility, which is one of four national NSF-funded supercomputer centres.

The NSF funds go to GA, a privately owned company involved primarily in nuclear research development, which employs the staff responsible for running the supercomputers. The computers themselves are housed in a building on UCSD's campus.

Differences in philosophy have led UCSD and GA to submit competing proposals for the next round of NSF funding for such centres, beginning in October 1997. This competition for NSF funding is designed to make supercomputers a more widely usable and diverse scientific asset.

The NSF's National Science Board does not want the \$65 million it pumps annually into the four supercomputer centres

to be 'an entitlement programme', say officials. About 10 potential bidders submitted 'concept papers' earlier this month. Final proposals are due in September.

As part of its proposal, UCSD is setting up a network linking all nine campuses of the University of California, as well as the Lawrence Livermore, Lawrence Berkeley and Los Alamos Laboratories which it runs for the Department of Energy, and several state universities.

"I'm very excited about it," says Robert C. Dynes, UCSD's newly designated chancellor. "Potentially, this is the library of the future," he says, referring to the network's information-transmission capabilities as "a blueprint for the nation".

Officials at GA decline to discuss their own proposal. N. Douglas Pewitt, a former government official who has been serving as acting director of the centre since mid-February, says that the company has submitted a one-page pre-proposal outlining its intentions.

According to observers, the split between GA and UCSD came about because of the nature of the new NSF funding programme. To meet the NSF's goals, UCSD wants to develop an academically led proposal with a broad network of partners, all of whom could capitalize on the use of the supercomputers. But GA saw its role in this model as being reduced merely to that of a computer operator, with limited overall control.

Both Pewitt and UCSD officials claim that they are continuing amicable discussions about a possible future combined bid for one of the new NSF grants. But the chances of such a bid succeeding are uncertain, given the personnel changes and other events of recent weeks.

In February, Sid Karin, the founding director of the centre, who had announced his resignation last autumn but had intended to stay on until 1 May, left GA suddenly to work on UCSD's proposal. Then, in March, GA officials told the university it planned to put in a unilateral bid for the NSF grant.

Shortly afterwards, the second and third most senior officials at the centre — Peter Arzberger and Wayne Pfeiffer — resigned, with Arzberger moving to UCSD's payroll to work on its proposal.

About the same time, GA officials used a ruse to get Karin's long-time secretary — who was resigning to join Karin at UCSD — out of her office. The company prevented her from returning, and then took control of her personal computer containing Karin's electronic mail and other information.

Following these events, an attorney representing GA sent Karin a letter that is said to have contained a veiled threat of legal action. Pewitt declines to comment on the letter. But a review of the situation, carried out by a retired GA executive and delivered at the beginning of this month, found that both the letter and efforts to monitor Arzberger's and Karin's e-mail have contributed to a plunge in morale among staff at the centre.

The review concludes that the legal threat was "very negative for the partners". Commenting on potential problems caused by the staff departures, it adds that "the respect they had for (centre) top management is hard to exaggerate".

The review also points out that, if GA moves ahead with its own competing proposal, there are likely to be further staff defections, particularly as the company's solo bid for the NSF contract is seen as having little chance of succeeding.

Richard E. Kaplan, an NSF contract officer for the facility, says he remains concerned that recruitment companies may take advantage of the uncertainty to strip the centre of more talent. "Headhunters are like sharks," he says. "They smell blood in the water."

Such fears were heightened last month when one of the centre's leading scientists — Hans-Werner Braun, a high-speed-network designer supervising substantial NSF grants — announced he was leaving to work at Teledesic Corporation, a company that is developing a satellite communications system.

Rex Dalton

Inquiry clears Canadian researchers

Ottawa. Three members of the engineering and computer science faculty at Concordia University, Montreal, who were forced out of the university in 1994 on suspicion of alleged financial irregularities, have been cleared after an investigation by the Natural Sciences and Engineering Research Council of Canada (NSERC), and their research grants have been restored.

The investigation found no evidence of misuse of its funds by M. N. S. Swamy, former dean of engineering, T. S. Sankar, then faculty coordinator of research, and Seshadri Sankar, who was director of the Computer Aided Vehicle Engineering Research Centre. The research council has reinstated their research funding at a level that puts them in the top five per cent of awards — provided that they can find eligible research positions.

The inquiry was undertaken after an audit ordered by the university had shown that its administration had permitted the use of NSERC funds for purposes that were not allowed. The three researchers

were mentioned in this audit, and this resulted in NSERC's freezing of their research grants and the university's general research grant.

The university then persuaded Swamy and T. S. Sankar to retire early and Seshadri Sankar to resign (see *Nature* **370**, 166; 1996). Concordia itself was required by NSERC to return the missing funds, and to set up new accountability and control mechanisms.

Since then, Swamy has been nominated by his colleagues as emeritus professor, and has again begun to conduct research at Concordia. The new rector, Frederick Lowy, says a return to the faculty by the Sankars is unlikely because of budget restrictions. But he suggests some arrangement may be found to permit them both — as well as Swamy — to resume research.

The Sankars themselves say they are confident the restoration of their research funds will make their attractive candidates for faculty positions at any university requiring their expertise. **David Spurgeon**

Superconductivity spurs Japanese plan for NMR research

Tokyo. A group of Japanese chemists and biologists, backed by the Science and Technology Agency (STA), has drawn up ambitious plans to establish a state-of-the-art centre for using nuclear magnetic resonance (NMR) to study the structure and function of proteins. The proposed centre would not only use the most advanced NMR machines available, but also aim to develop new NMR technology using high-temperature superconductors.

The group is led by Akiyoshi Wada, former dean of science at Tokyo University, who is said to have won backing at the highest level in STA. As a result, the first funds for a feasibility study are likely to be included in the agency's budget request for fiscal year 1997 (which starts on 1 April), due to be submitted at the end of August. In Japan, commitment to such a study almost invariably leads to implementation of the project.

The centre, or 'NMR Park' as it is called, would initially use 750–800 MHz NMRs, the most powerful machines available which use conventional superconducting magnets. But, in addition to carrying out research with these machines, another aim of the park would be to develop much higher resolution 1 GHz NMRs, using coils of both conventional and high-temperature (high- T_c) superconductors.

Japan is advanced in the development of high- T_c superconducting wires, and is in a strong position to develop such coils. But Yoji Arata, a former professor of physical chemistry at Tokyo University who is backing the project, admits that it may take some time before it is possible to achieve the necessary resolution and stability with high- T_c coils.

Supporters of the park want it to be a truly international centre drawing scientists from around the world, much like Japan's 'Photon Factory', a synchrotron facility in Tsukuba science city. Arata will host an international symposium for structural biologists and NMR specialists later this month in Tsukuba at which he hopes to enlist support and advice from overseas leaders in the field.

Funds for the project would be channelled through the Institute of Physical and Chemical Research (RIKEN), which is expected formally to endorse the project later this month. A site for the park has not yet been chosen, and it will take until at least the end of this year before STA can set a firm budget. But a figure of ¥50 billion (US\$500 million) for the overall cost of the park is being widely quoted.

David Swinbanks

'Race and IQ' psychologist in inquiry over teaching conduct

London. The University of Edinburgh has launched an inquiry into the teaching conduct of Chris Brand, a lecturer in psychology at the university, and author of a controversial book on race and intelligence that was withdrawn last month by its US publishers, John Wiley and Sons.

The inquiry — seen by Brand as signalling the encroachment of 'political correctness' in British universities — will focus on his teaching methods, following complaints from students that he made them feel "uncomfortable" during classroom discussions with his contentious views on race, gender and intelligence.

In his book, *The g factor: General Intelligence and its Implications*, Brand claims that the genetic component in intelligence outweighs environmental influences, and repeats the view that blacks in the United States obtain lower average IQ scores than whites.

The publishers withdrew the book after newspaper interviews quoted Brand as having described feminism as a 'menace' and suggesting that single mothers ought to mate with males of a higher IQ. A statement from the publishers described Brand's assertions as "repellent" (see *Nature* 381, 10; 1996).

The inquiry was announced at a press conference by Sir Stewart Sutherland, principal of the university. It will be carried out by Neil MacCormick, dean of social sciences and professor of law at the university, who will report to the principal next week.

In a statement, Sutherland, a former vice chancellor of the University of London, said he had "no hesitation" in saying that "the large majority of my colleagues in the university do not share conclusions on eugenics apparently held by Mr Brand". Many of Brand's students continue to boycott his classes. Sutherland also announced plans for a seminar later in the term, to include speakers "with various viewpoints on intelligence and the relative importance of heredity and environmental factors".

Brand describes the inquiry as a "side issue" that is detracting attention from "the suppression" of his book. The question of his teaching methods, he says, would not have arisen were it not for the publicity surrounding the book. He vigorously defends his teaching style which he describes as "frank and relaxed".

"Students are simply the victims of the

religious hysteria of our times", he says. "This is why they find everything that touches on their prejudices to be insensitive. If in deference to 'sensitivity', I draw a veil [on controversial issues], they'll find me incomprehensible."

Meanwhile, Brand continues to respond to the controversy he has generated with vigour. Indeed, the worldwide publicity generated by Wiley's decision to distance itself from the book appears to have had unexpectedly favourable consequences. The lecturer claims to have several offers to publish the book, and says any new publisher is likely to print and sell many more than the 2,000 copies planned by Wiley.

Brand has already demanded that Wiley pay US\$75,000 for a relaunch. The alternative, he states, is for Wiley to apologize publicly, resume publication and supply psychology libraries worldwide with a free copy of the book. The publishers, according to Brand, have merely offered to provide him with unjacketed copies of the book, and to reassign the copyright.

Brand's decision to differentiate his position on race and intelligence from the withdrawal of the book is a calculated one. It has put many of his scientific opponents, as well as his university, in the position of having to support the right to 'academic freedom'. Indeed, many would prefer his book to be published so that his 'scientific' arguments could be held up to academic scrutiny.

The book itself presents a chain of assertions: that IQ is a measure of intelligence; that intelligence boils down to a measure of the speed of apprehension, that intelligence is largely governed by genetic factors, and that all this has implications that should be taken into account by society, particularly in formulating education policy.

Critics have challenged Brand's position by attacking the assertions he makes to hold it up. For example, Nicholas MacIntosh, professor of experimental psychology at the University of Cambridge, who reviewed the book last week (see *Nature*, 381, 33; 1996), says that the least academically sound of his assertions is that the g factor — for 'general intelligence' — can be reduced to a measure of speed of apprehension.

MacIntosh says that Wiley's response was unwise. "I don't think what they find offensive is new", he says. "On the whole I believe that academics ought to be able to publish what they believe in." Wiley declines to add to a statement issued when the book was withdrawn: "John Wiley Ltd does not want to support these views by disseminating them or be associated with a book that makes assertions that we find repellent."

Ruth Bell & Ehsan Masood



Brand: defiant of student boycott.

Research council heads to review Europe's research effort

Munich. The organization of heads of European research councils, known as Eurohorcs, is to carry out an analysis of the strengths and weaknesses of European research, using international groups of experts to help to assess about ten scientific areas. The exercise aims to identify research areas which could benefit from greater collaboration in Europe and where, for example, centres of excellence might be established.

Although the Eurohorcs, at a meeting in Vienna last month, did not decide on the criteria to be used for assessing research fields, they still agreed — some feel rather optimistically — that they would aim to complete the task within six months, so as to provide information to the European Commission before the first draft of the commission's fifth Framework programme of research is completed at the end of the year. The steering committee will be headed by Eurohorcs' president, Wolfgang Frühwald from the Deutsche Forschungsgemeinschaft (DFG). The European Science Foundation in Strasbourg will help to identify individuals to make up international panels of experts for each category. □

Observatory change 'risky'

Washington. Managers of the US National Optical Astronomy Observatories (NOAO) say they will have to close the 4-metre Mayall Telescope on Kitt Peak, Arizona, in addition to several smaller telescopes previously targeted for closure there and in Chile (see *Nature* 379, 569; 1996). The NOAO restructuring plan calls for building new instruments in both hemispheres, including the twin Gemini 8-metre telescopes. The Mayall would close or be privatized in 1999.

The proposal to close the Mayall — the largest telescope on Kitt

Peak — was prompted by the increasingly bleak outlook for astronomy funding at the National Science Foundation, which pays the Association of Universities for Research in Astronomy (AURA) to run the NOAO. In a letter posted on the World Wide Web, AURA managers say: "The difference between level dollar funding and funding adjusted at the rate of inflation is the difference between operating and not operating the 4-m Mayall telescope."

The director of NSF, Neal Lane, in a letter of 5 April to AURA president Goetz Oertel, acknowledged that the restructuring is a "risky experiment", and that the NSF had little desire to see productive telescopes closed. But "in the present budgetary climate we have little alternative". The NOAO users' committee last week urged astronomers to write to the NSF, and warned that "a continuation of the last 10 years of declining NOAO budgets will eventually destroy the science programs of astronomers across the United States". □

Fungi species head for oblivion

London. Fungi are becoming extinct faster than scientists can study them, according to a declaration issued by 85 leading international mycologists at the British Mycological Society's centenary symposium at the University of Sheffield last month. The declaration expressed deep regret at the continued loss of habitat for fungi around the world and called for action to cut the rate of loss. Of the estimated 1.5 million species of fungi in the world, only 72,000 have been described by scientists. □

Australian academics warn of cuts

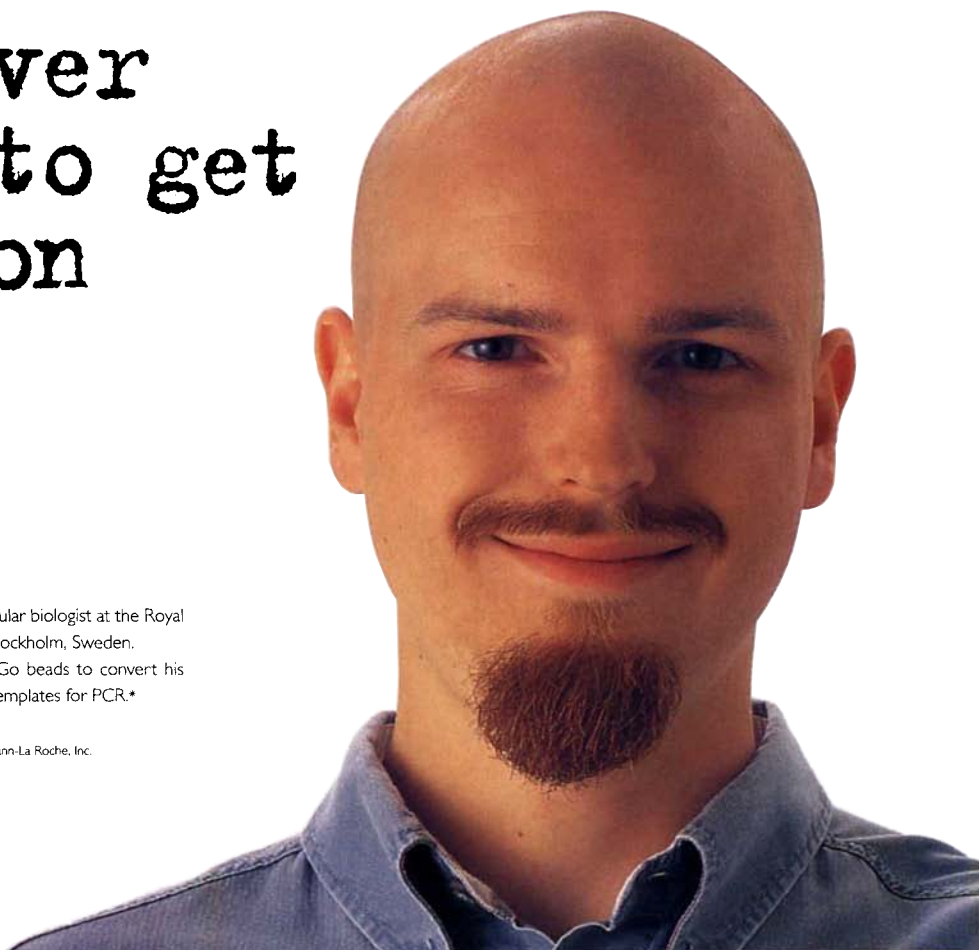
Sydney. Research leaders at Australian universities are stepping up a public campaign against threatened cuts to research funding, claiming in a statement issued last week that "the consequences for the economy would be dire if the government failed to honour its election promises". They warn that, if infrastructure for research is

Patrik never fails to get a reaction

Patrik Samuelson is a molecular biologist at the Royal Institute of Technology in Stockholm, Sweden.

Patrik uses Ready-To-Go beads to convert his RNA samples into cDNA templates for PCR.*

* PCR is a patented process of Hoffmann-La Roche, Inc.



Academic management

SIR — There seems to be a general misconception among scientists about decision-making committees such as search committees and review boards.

Filling an academic vacancy is a management task where scientific performance is just one of many criteria leading to the selection of a candidate. Whether the use of impact factors is sufficient to evaluate scientific performance¹ or not^{2,3}, other factors have to be taken into account as well. Whereas these explicit academic management objectives are more or less well defined and intuitive (scientific maturity, teaching skills, grant application proficiency), it is the implicit objectives⁴ the scientific community has to be wary of.

It is misleading and frustrating, especially for young scientists, that within scientific disciplines it is assumed that academic promotion will follow processes that are somehow related to the inference mechanisms in science itself. Given the same dataset, it is assumed, we arrive at the same conclusions. It is trite to say that neither science nor appointments work that way. But it is exactly the different interpretation of the applicants' performance data that provides an excellent mask for the personal objectives of committee members.

It has been pointed out that personal objectives can result in favouritism, and although many countries require advertising of vacant academic positions in scientific journals, and although equal opportunity is usually assured, the null hypothesis that "local candidates are equally or less likely to be appointed than others" (where "local" should be carefully defined) will have to be rejected^{1,5}. Unfortunately, university regulations or law in many countries require advertising even for positions that are in fact already bestowed.

In my opinion, two points have been disregarded in the discussion so far. First, a tied (or split) decision within a decision-making committee results in trade-offs among power factions, which in turn leads to the promotion of the candidate who represents the lowest common denominator. And second, the aversion factor may even render scientific excellence detrimental to selection. A qualified scientist or teacher can be highly motivating for many of his or her colleagues and at the same time threaten the establishment by questioning authority of, in particular, two kinds of scientists: the superficially successful scientist whose research is run by low-level members of his or her research team, and, the scientist whose tenure, obtained a long time ago, preserves a chair in an office in case he or she cannot sit on a committee. Neither of them has a vital interest in the appointment of a troublemaker (and having a critical

letter published in *Nature* qualifies as such). Unfortunately, it is these two kinds of scientists who can spend their time lobbying on their own behalf. And let's not forget, the members of these committees exert power. Change in academic management is unlikely and can come only from within the scientific community itself. Until then we are stuck in a tragedy of the academics in Hardin's sense⁶.

Academic hiring is already prone to abuse when evaluation procedures of applicants' achievements are clearly outlined, as reported by Gaetani and Ferraris¹. The potential for arbitrariness increases when there are no guidelines for decision-making committees. After questioning the selection process for an instructor position at the department in which I am working, a review committee concluded that: "It is a measure of our informal atmosphere and congeniality that we lack formal criteria..."

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1. Gaetani, G. F. & Ferraris, A. M. *Nature* **353**, 10 (1991).
2. Metcalfe, N. B. *Nature* **376**, 720 (1995).
3. Lewison, G., Anderson, J. & Jack, J. *Nature* **377**, 671 (1995).
4. Walters, C. *Adaptive Management of Renewable Resources* 1–374 (Macmillan, New York, 1986).
5. Perez-Enciso, M. *Nature* **378**, 760 (1995).
6. Hardin, G. *Science* **162**, 1243–1248 (1968).

Neem pest not a mystery

SIR — There is no mystery about the insect responsible for severe damage to neem trees¹. It is a mirid bug² called the 'tea mosquito' (*Helopeltis antonii* Signoret, Heteroptera; Miridae), confined to South India, Sri Lanka and the Andaman Islands. The nymphs and adults suck the sap from the shoots, resulting in lesions that coalesce and become necrotic with gummosis. The shoots eventually wilt and the infested tree has a burnt-up appearance. This phenomenon is seen every year in the state of Tamil Nadu. The insect has a host range of more than 35 species belonging to 24 plant families that include commercial crops such as cashew, cocoa, tea and guava.

Neem is an evergreen tree in which flushes of new leaves with panicles appear in February–March and fruits mature in June–July. New flushes continue after the fruiting season until September. Even though *H. antonii* breeds continuously on neem, the outbreak occurs during the non-flushing season (November–December) in the hinterland. The affected tree maintains its burnt-up appearance until the following February. In coastal areas, there is a minor outbreak in October–November.

The major outbreak occurs in summer (March–April) on new flushes which rejuvenate after a month. In both regions, however, productivity of neem seed is affected in the infested trees.

While feeding, *H. antonii* injects toxic saliva into the plant, causing phyto-toxaemia. From the salivary glands of the insect, we have detected hydrolytic enzymes (protease and lipase), and oxidoreductase enzymes (catechol oxidase, peroxidase and catalase). These enzymes³ cause phyto-toxaemia as well as detoxification of plant chemicals that defend against herbivores. Moreover, free amino acids present in the saliva of *H. antonii* interfere with plant defences and protect digestive enzymes of the saliva from denaturation⁴. This mechanism allows the insect to overcome the defensive chemicals present in neem and to establish itself as a primary pest and cause severe damage.

Three species of endoparasitic wasps, *Ufens* sp. (Trichogrammatidae), *Erythmelus helopeltidis* Gahan (Mymaridae) and *Telenomus* sp. *laricis* group (Scelionidae) attack *H. antonii* eggs in the remaining part of the year, thereby limiting damage to new leaves.

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1. *Nature* **378**, 532 (1995).
2. Rao, Y. R. *Agric. J. India* **10**, 412–416 (1915).
3. Miles, P. W. & Peng, Z. *J. Insect Physiol.* **35**, 865–872 (1989).
4. Laurema, S. & Varies, A. L. *Insect Biochem.* **21**, 759–765 (1991).

Ads from the edge

SIR — I rarely read your advertisements, but the paid advertisement "Marinov: Annus Horribilis" (*Nature* 28 March 1996) caught my attention. Marinov's treatise appears to debunk most of the orthodox science you regularly publish.

That raises the following questions: can I bypass the *Nature* review process by buying a paid advertisement for questionable scientific theories? What are your editorial standards for advertisements appearing in *Nature*?

If this letter does not appear in the Correspondence pages in the near future, I shall publish it as a paid advertisement.

Kofi Crentsil

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■ *Nature* has from time to time nodded in the direction of freedom of speech by allowing unorthodox science into its advertising pages. This permissiveness will continue to be whimsical. — Editor, *Nature* □

Lakes under a three-pronged attack

Evlie Gorham

Two studies of environmental damage to lakes in Canada teach us several lessons — not least, that understanding such problems requires long-term investigations that take into account the complex interactions involved.

THE penetration of lake waters by harmful ultraviolet radiation is related to climate warming and acid rain, as well as to depletion of stratospheric ozone, according to two papers published this year in *Nature* by Schindler *et al.*¹ and (on page 141 of this issue) by Yan *et al.*². The interactions between these environmental stresses are complex and are of exceptional interest because environmental problems are seldom subjected to this sort of analysis.

Humans, by their various activities, have made many inadvertent 'experiments' that have had serious consequences for natural ecosystems. Unfortunately, they have lacked the controls that are an important part of truly scientific experiments. Vast amounts of carbon dioxide and sulphur dioxide have been released to the atmosphere through the combustion of fossil fuels and the smelting of metal-sulphide ores. These releases threaten to alter ecosystems in a variety of ways: through global warming, an increased frequency of droughts, acid rain and the direct toxic effects of sulphur dioxide on terrestrial plants. The release of industrially produced chlorofluorocarbons is another such inadvertent experiment, which has caused serious concern owing to its depletion of ozone in the stratosphere, increasing the exposure of organisms to biologically damaging UV-B radiation.

The effects of such radiation on lakes range from inhibition of photosynthesis by phytoplankton to sunburn patterns on trout — coupled with increased fungal infection and death rates. Scientists study such accidental experiments both to mitigate their ill effects and to learn how ecosystems function. They also perform their own deliberate experiments, manipulating ecosystems directly to investigate such matters.

Schindler *et al.*¹ added sulphuric acid to lakes in the Experimental Lakes Area of northwestern Ontario that they had been studying and in some cases manipulating for two decades. They have shown that both acid levels and climate warming over the past 20 years have led in different and complex ways³ to declines in dissolved organic carbon (DOC) compounds in lake waters. These coloured aromatic compounds absorb solar radiation, including UV-B, and their decline has permitted it to penetrate much deeper into the water. The effect was particularly evident in

clear, shallow lakes, which are common in the boreal zone (the huge northern temperate zone of coniferous forests) and even more so in arctic and alpine regions, and it outweighed the effect of ozone depletion in exposing aquatic organisms to damage by UV-B radiation. Schindler and colleagues noted further that in such lakes some of the biological damage attributed to acid deposition may instead have been caused by increased UV-B exposure.

Many lakes throughout the boreal zone, like this one in Ontario, Canada, may be losing their natural shielding to ultraviolet radiation.

Now Yan *et al.*² report on a similar situation in Swan Lake, the subject of an 'experiment' near Sudbury in northern Ontario, where the smelting of copper and nickel ores devastated the landscape for several decades, although the damage has abated considerably in recent years. Swan Lake has been subjected to severe atmospheric deposition of sulphur from smelter fumes, and substantial amounts of it have been stored in sediments. During a two-year drought in 1986 and 1987 the water level dropped considerably, so that the lake's surface area shrank by 18 per cent. This led to oxidation of the sulphur compounds contained in the uppermost layer of exposed near-shore sediments to sulphuric acid. In the subsequent wet year the acid washed into the lake and lowered its pH greatly, from 5.8 before the drought to 4.5 in the year after. DOC declined by a factor of three, in part directly by acidifi-

cation and in part by settling out after aggregation with sedimentary aluminium released by the acidification. Because of the decline in DOC, the depth of the lake exposed to at least 1 per cent of surface UV-B radiation increased from 1.8 m in 1987 to 5.6 m in 1988, corresponding to 94 per cent of lake volume.

We see in these two kinds of experiment that three anthropogenic stresses on the environment — climate warming, acid

deposition and ozone depletion — are linked to one another through their influence in deepening the penetration of UV-B radiation into clear-water lakes. It is likely, moreover, that this influence will increase as ozone depletion worsens, climate continues to warm and sulphur compounds, stored in lake sediments and wetland peats as a result of more than a hundred years of acid deposition, oxidize in response to falling water tables. Further reduction of sulphur emissions becomes, therefore, a more urgent matter

1. Schindler, D. W., Curtis, P. J., Parker, B. R. & Stainton, M. P. *Nature* **379**, 705–708 (1996).
2. Yan, N. D., Keller, W., Scully, N. M., Lean, D. R. S. & Dillon, P. J. *Nature* **381**, 141–143 (1996).
3. Schindler, D. W. *et al. Hydrobiologia* **229**, 1–21 (1992).
4. Gorham, E., Underwood, J. K., Martin, F. B. & Ogden, J. G. *Nature* **324**, 451–453 (1986).
5. Tilman, D. in *Long-Term Studies in Ecology: Approaches and Alternatives* (ed. Likens, G. E.) 136–157 (Springer, New York, 1989).
6. Nikiforuk, A. *Toronto Globe and Mail* D8 (9 March 1996).

Nigel Tucker/Planet Earth Pictures

than has hitherto been thought.

Yan *et al.* state that rapidly flushing lakes with wetlands in their catchment areas are those most at risk, particularly in regions where acid deposition by rain and snow is severe. Wetlands do, however, provide considerable mitigation of UV-B penetration by exporting dissolved organic carbon to lakes⁴. It remains to be seen whether the oxidation of their stored reduced sulphur will offset, by further acidification, the influence of such carbon export.

These fascinating studies reinforce several concerns that have been expressed by people and organizations that have the welfare of our environment at heart, and

teach us a few lessons. First, most of our diverse impacts on the environment are studied as separate problems, and only rarely do scientists examine appropriately their many and complex physical, chemical and biological interactions. Second, environmental problems require for their proper understanding long-term investigations lasting decades. Such studies occur all too seldom⁵. Third, long-term manipulative experiments are of immense value in validating shorter-term comparative studies. Fourth, research on one environmental stress can prove most helpful in elucidating the consequences of other distinctly different stresses.

It is clear that long-term data sets can have all sorts of unanticipated uses if they are the product of thoughtful and careful design. The value increases steadily, and more than additively, as the period of study increases. In our rapidly changing world it is of vital importance that such studies be allowed to continue without suffering the serious reductions in support threatened for the Experimental Lakes Area⁶. □

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HUMAN GENETICS

Mutation-causing mutations

Nathan A. Ellis

THOSE of us engaged in positional cloning of genes often wonder what 'our' gene is going to be like: one dreams of just the right mix of novelty and informativeness. The fulfilment of such a wish is to be found in a paper by Yu *et al.* published last month in *Science*¹ — the authors have cloned the gene which, when defective, causes Werner's syndrome, and find that it is a member of the RecQ helicase family of proteins. The finding is the second time in human genetics that mutation in a RecQ homologue has been linked with a mutator phenotype.

Werner's syndrome (WS) is a rare autosomal recessive disorder, features of which include early onset of cataracts, generalized hair loss, loss of skin elasticity, osteoporosis, atherosclerosis and arteriosclerosis, and short stature from childhood. In all, WS patients give the impression of having aged prematurely, prompting the notion that the disease is a model for the normal ageing process. Thirty years ago, however, Epstein *et al.*² pointed out that the syndrome "may be better considered a 'caricature' of aging, exaggerating although not necessarily by the same mechanisms, some of the clinical and pathologic features which connote aging".

In the same paper², these authors speculated that a fault in a DNA-repair mechanism in non-proliferative tissues or tissues regularly exposed to stress could be a key factor in WS. Subsequent cytogenetic analysis uncovered a most unusual characteristic, known as 'variegated translocation mosaicism' — cells carrying different non-constitutional karyotypic abnormalities (inversions, translocations, chromosome losses) were found in short-term cultures of dermal fibroblasts and of peripheral lymphocytes^{3,4}. Consistent with this, the mutation rate in SV40-transformed WS fibroblasts is some 50

times higher than in non-WS control cells, the predominant form of mutation being gross DNA deletions⁵; DNA replication is also disturbed, in that the distance between sites of replication initiation is increased⁶. The increased chromosomal and locus-specific mutations translate into increased probability of developing tumours: WS patients often get non-epithelial tumours and, to a lesser extent, leukaemia and carcinoma (see ref. 7 for review).

The increasingly potent technologies of positional cloning have now permitted the isolation of the gene (*WRN*) for Werner's syndrome within only four years of its localization to chromosome 8 by homozygosity mapping⁸. Yu *et al.*¹ analysed the DNA of families with a history of WS to identify recombinants, and were able to restrict their search to a region of one million base pairs. They then looked for

mutations by direct sequencing of some ten genes in that region, and alighted upon *WRN* as a candidate. They identified four different mutations in the gene, all of them leading to truncation of the predicted protein and presumably to loss or deficiency of function. One of the mutations, a splice-junction base change that resulted in exon-skipping, occurred in 60 per cent of Japanese WS patients. This confirmed work that had shown a relatively increased frequency of WS in the Japanese, and linkage disequilibrium between *WRN* and adjacent markers in Japanese WS patients — all together indicative of a founder effect caused by genetic drift.

But the biggest surprise by far came from database comparisons, which showed that the *WRN* product is most similar to the RecQ subfamily of DEXH-box-containing helicases. Helicases unwind nucleic-acid duplexes to provide access of template to protein factors. Like other helicases, the RecQ subfamily, which now includes members from *Escherichia coli*, yeast, nematode and human, contains seven conserved amino-

Selected features of Werner's and Bloom's syndromes

	Werner's syndrome	Bloom's syndrome
Genetics	Autosomal recessive	Autosomal recessive
Prevalence	Rare (except in Japanese)	Rare (except in Ashkenazi Jews)
Clinical features*	Short stature Neoplasia Hypogonadism† Normal intelligence	Short stature Neoplasia Hypogonadism† Normal intelligence
Cytogenetic abnormalities	Variegated translocation mosaicism (see text)	Increased chromatid exchange Increased chromosome breakage
Mutation rate	Increased (deletions) Hyper-recombination†	Increased? Hyper-recombination
DNA replication	Altered distance between initiation sites	Decreased elongation rate Abnormal intermediates
DNA repair	Normal	Normal
Protein homology	RecQ helicases	RecQ helicases

* The features listed are not exhaustive, and there are dissimilarities between the two syndromes. See refs 2 and 10 for details.

† Subfertility in WS; infertility in BS males and subfertility in BS females.

‡ Hyper-recombination in WS was detected between extra-chromosomal plasmids transfected into WS cells (a single report).

acid motifs. More specifically, where it is known, the RecQ helicases encode non-essential activities that operate on DNA rather than RNA, and are central in the maintenance of genomic stability.

In human genetics, genetically determined chromosome instability — chromosome breakage — was first discovered in the 1960s in Bloom's syndrome (BS). In a sense, BS is the prototype of a group of rare disorders which are characterized by genomic instability and which include ataxia-telangiectasia, Cockayne's syndrome, Fanconi's anaemia, Lynch syndromes I and II, WS, Wiskott-Aldrich syndrome and xeroderma pigmentosum. In all of these disorders, distinct chromosomal and locus-specific mutations arise much more frequently than usual, spontaneously or in response to environmental agents (or both). They can be classified as somatic mutational diseases, of which cancer itself is an important constituent.

Before *WRN*, the most recent addition to the RecQ subfamily was the product of the Bloom's syndrome gene, *BLM* (ref. 9). Some features of BS bear uncanny resemblance to those of WS (see table). BS cells exhibit an increased rate of somatic mutations and chromosomal abnormalities, as do WS cells, and people with BS are highly predisposed to cancer of the sites and types that affect the general population¹⁰. A key difference is that people with WS incur disproportionately more non-epithelial cancers (ref. 7); the restriction in type of cancer perhaps reflects a restriction in the expression of *WRN* to non-epithelial tissues, as observed by Yu *et al.* This difference notwithstanding, identification of the *WRN* product as homologous to RecQ helicases brings WS and BS closer together than ever before.

Within the RecQ subfamily, two groups have emerged. One is represented by RecQ itself. Mutations in the *E. coli recQ* gene are neither mutators nor hyper-recombinogenic; the protein is implicated in post-replication recombinational repair and contains 609 amino acids. Human RECQL (for RecQ-like) protein, function unknown, is similar to RecQ in amino-acid length.

The other group is represented by

WRN, *BLM* and *SGS1*, a yeast RecQ homologue. Mutations in each of these genes cause mutator genotypes, including hyper-recombination and chromosome instability. The gene products are similar in length (just over 1,400 amino acids), and their amino-acid compositions outside the domain containing the helicase motifs are strikingly similar, being negatively charged at the amino terminus and positively charged at the carboxy terminus. These properties suggest that these proteins have similar functions in the maintenance of DNA stability.

The characteristic chromosomal abnormalities in WS and BS cells, and the defects in initiation of DNA synthesis and chain elongation, respectively, imply that the *WRN* and *BLM* gene products are involved in DNA replication. Although DNA repair appears to be functioning normally in both WS and BS cells, it is possible that there is a hitherto undiscovered defect in repair. Faulty DNA helicases have been identified in xeroderma pigmentosum and Cockayne's syndrome, disorders in which DNA repair is defective. In any case, in the absence of the

WRN and *BLM* helicases somatic cells accumulate mutations.

DNA helicases play multifarious roles in DNA metabolism, including replication, transcription, repair and recombination. In these processes, the helicases interact with other factors that regulate their activity in carrying out helicase function: to unwind the DNA duplex to provide access for other factors. The key questions now are in which processes do the *WRN* and *BLM* helicases participate, with what factors do they interact, and what are the specific substrates they operate on? With the isolation of the WS and BS genes, new reagents and assays can be devised to address these questions. The *WRN* and *BLM* helicases are quite possibly the vanguard of a large family of RecQ helicases in the human genome, the function of which is to help maintain genomic stability. □

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GAIA

The physiology of the planet

Lee R. Kump

ARE important environmental variables like temperature and atmospheric composition regulated by feedback between the biota and the physical world? Is the Earth habitable today only because these feedback mechanisms have evolved with the biosphere over its 3.5-billion-year history? These were the fundamental questions addressed at last month's Gaia meeting*. Although no clear answer emerged to the latter question, a number of papers presented convincing evidence for self-regulation. Indeed, new insights provided by the 'geophysiological' approach provided much of the impetus for the creation of a new scientific society, the Geophysiological Society, which held its first business meeting at this conference.

The physiological analogy became a major theme early in the meeting, following the presentation of a study that used Watson and Lovelock's 'Daisyworld' model¹ to investigate the human regulation of blood glucose levels (J. Koeslag, Univ. Stellenbosch, South Africa; P. Saunders, Kings College London). On Daisyworld, two species of daisy, white and black, control the temperature of the planet by their relative abundance. White daisies increase in numbers if the planet begins to overheat, reflecting sunlight and

cooling it down again; black daisies do the opposite for an over-cool planet. Between them they act as a negative feedback mechanism and stabilize the environment.

Similarly, two hormones regulate glucose levels in human blood: glucagon, which stimulates glucose production; and insulin, which stimulates glucose utilization. Faced with higher blood-glucose levels, the body immediately generates large quantities of insulin. This acute response is followed by a gradual decline in insulin levels to a new equilibrium concentration, higher than the initial level, but lower than that achieved immediately after the glucose increase. In contrast, glucagon concentrations fall slowly to the new equilibrium. The acute response is an over-reaction, which is eventually corrected.

Does the Earth system show such behaviour? Several candidates were discussed, including the large pulse of oxygen production that apparently coincided with the establishment of an oxygen-rich atmosphere some 2 billion years ago (H. D. Holland, Harvard Univ.), and the widespread glaciation that occurred some 440 million years ago at a time of otherwise global warmth. Was this glaciation an acute response to the early colonization of the land surface? Most geochemists agree that forests promote the chemical weathering of the crust, a process that removes carbon dioxide from the atmosphere and

1. Yu, C.-E. *et al.* *Science* **272**, 258–262 (1996).
2. Epstein, C. J., Martin, G. M., Schultz, A. L. & Motulsky, A. G. *Medicine* **45**, 177–221 (1966).
3. Hoehn, H. *et al.* *Cytogenet. Cell Genet.* **15**, 282–298 (1975).
4. Scappaticci, S., Cerimele, D. & Fraccaro, M. *Hum. Genet.* **62**, 16–24 (1982).
5. Fukuchi, K.-I., Martin, G. M. & Monnat, R. J. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5893–5897 (1989).
6. Takeuchi, F. *et al.* *Hum. Genet.* **60**, 365–368 (1982).
7. Goto, M., Miller, R. W., Ishikawa Y. & Sugano, H. *Cancer Epidemiology, Biomarkers, and Prevention* **5**, 239–246 (1996).
8. Goto, M., Weber, J., Woods, K. & Drayna, D. *Nature* **355**, 735–738 (1992).
9. Ellis, N. A. *et al.* *Cell* **83**, 655–666 (1995).
10. German, J. *Medicine* **72**, 393–406 (1993).

*Gaia in Oxford II: The Evolution of the Superorganism, Green College, Oxford, UK, 30 March – 3 April 1996.

Controlling the daisies: James Lovelock mowing his lawn in Cornwall. Lovelock has championed a new scientific approach, geophysiology, that views life and the physical world as part of a global system capable of environmental regulation. Unlike the original Gaia hypothesis, geophysiology imposes no teleological demands on the biota. Self-regulation arises as a natural consequence of biota-environment interactions.

thus tends to reduce the greenhouse effect and global temperature². Perhaps the early colonization, by bryophytes and other primitive land plants, of a land surface unaccustomed to biological weathering generated a pulse of chemical weathering and an acute reduction of atmospheric CO₂. This was followed by an adjustment in CO₂ sources and sinks, and ultimately an equilibrium was reached with somewhat lower atmospheric CO₂ concentrations and cooler global climates.

The physiological analogy also seems to apply to the history of the Gaia hypothesis. Lovelock and Margulis developed the original hypothesis^{3,4} in response to the lack of consideration by their peers of an active role for the biota in the regulation of environmental variables. The original hypothesis, which postulated that organisms regulate the planet for their own advantage, was an acute response, an overreaction, and has undergone a series of adjustments leading to the new idea of geophysiology. Geophysiology emphasizes global self-regulation as a natural consequence of the interactions between the biota and the physical world. Self-regulation demands no foresight or planning, as the original Gaia hypothesis seemed to. What it does require is for biological activity to exert a substantial influence on the physical environment, and vice versa. While the recognition of both of these sets of effects is ingrained in the physical and life sciences, the consequence, self-regulation, is not.

Several participants expressed the view that, to the extent that the geophysiology describes organisms interacting among themselves and with their environment at a global scale, and recognizing that these interactions lead to self-regulation, one can consider the system a superorganism

(L. Margulis, Univ. Massachusetts). But could the present set of stabilizing feedbacks evolve through natural selection? While not denying the existence of self-regulation, the sceptical viewpoint sees no selective advantage for those organisms that contribute to global stability (J. Maynard Smith, Univ. Sussex). However, the progression from organisms that gained local advantage by altering their immediate environment to global self-regulation may have been stepwise, not unlike the steps that led from phototropism in individual cells to the evolution of a functional eye (T. Lenton, Univ. East Anglia; J. Lovelock, Coombe Mill, Cornwall).

Organisms that influence the global environment, like coccolithophorids, tiny marine animals that influence cloudiness by their emissions of dimethyl sulphide (DMS), or trees, which promote weathering, all perform their vital functions for other reasons. Coccolithophorids produce DMS as a by-product of osmoregulation, and trees create acidic soils to acquire mineral nutrients. These selective advantages have allowed the organisms to thrive. As their populations soared, so too did their impact on the environment; the

feedback loop achieved closure. Some of these feedback loops are destabilizing, but they probably feed into other loops that lend stability to the system. The DMS-climate feedback appears to be positive today, so climatic stability probably relies on stabilizing factors such as the tree-weathering feedback⁵.

There may be other evolutionary paths towards stability than the stepwise one of Lenton and Lovelock. The order of an ant colony seems to arise when population densities reach a critical level — below this level the movement of individual ants is chaotic (B. Goodwin, Open University). And geophysiology may provide testable hypotheses. As organisms merge to form superorganisms, parts which served a function for the individual organism may no longer be needed, and should be lost through selection (D. McShea, Santa Fe Inst.). Gaia is healthy in its new manifestation as geophysiology, and the prognosis is for a long and fruitful life yielding many deep insights into the functioning of the Earth system. □

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1. Watson, A. J. & Lovelock, J. E. *Tellus* **35B**, 284–289 (1983).
2. Holland, H. D. *The Chemistry of the Atmosphere and Oceans* (Wiley, New York, 1972).
3. Lovelock, J. E. *Atmos. Envir.* **6**, 579–580 (1972).
4. Lovelock, J. E. & Margulis, L. *Tellus* **26**, 1–9 (1974).
5. Lovelock, J. E. & Kump, L. R. *Nature* **369**, 732–734 (1994).

TRANSCRIPTION

Picking up the TAB

Stephen K. Burley

IN eukaryotes, RNA polymerase II (pol II) is responsible for transcribing nuclear genes encoding the messenger RNAs and several small nuclear RNAs. Like RNA polymerases I (ribosomal RNA transcription) and III (5S RNA and transfer RNA), pol II cannot recognize its target promoter directly. Instead, transcription initiation by pol II is precisely regulated by proteins called transcription factors that interact with promoter DNA targets and also with each other.

A number of structural biologists have been worrying away at this complicated problem of molecular recognition, and their latest contribution to solving it comes in papers published on page 127 of this issue¹ and in this week's *Science*². Tan *et al.*¹ and Geiger *et al.*² have carried out X-ray crystallographic studies of a complex of transcription factor IIA (TFIIA), the TATA-box-binding protein (TBP) and promoter DNA, and have re-

solved the structure at 2.5 Å and 3.0 Å, respectively.

Many other proteins interact with class II nuclear-gene promoters during transcription catalysed by pol II. The general initiation factors TFIIB, -D, -E, -F and -H must assemble on the promoter with pol II before transcription begins. TFIID is the only one of these components with sequence-specific DNA-binding activity (provided by its TBP subunit).

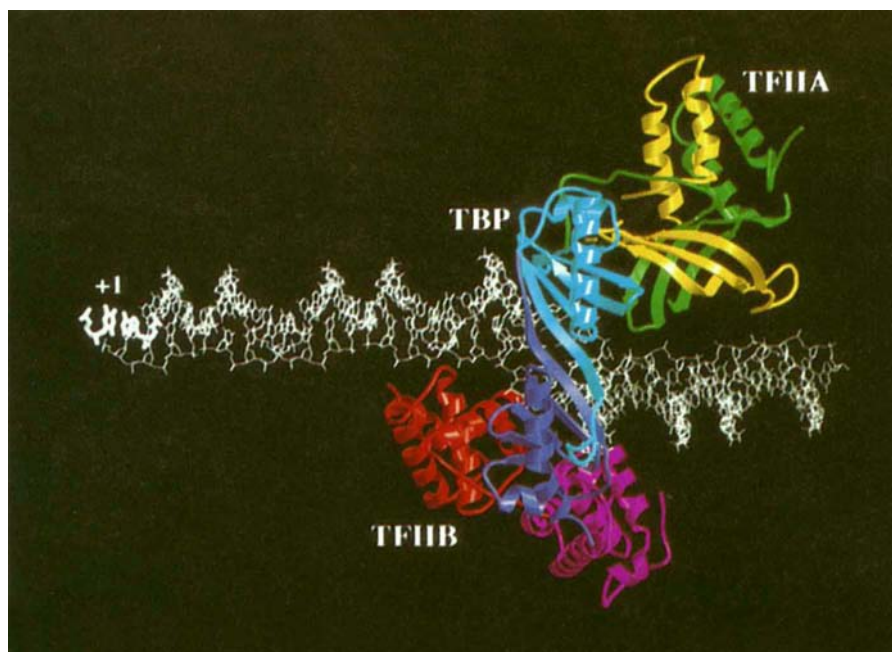
In the most general case, the orderly process of transcription initiation begins with the TBP subunit of TFIID recognizing and binding tightly to the TATA consensus sequence (TATAa/tAa/t). Thereafter, the TFIID-TATA box or D complex directs accretion of the remaining general initiation factors and pol II to form the preinitiation complex, a large multiprotein-DNA assembly that supports accurate initiation of transcription at one or at most a few start sites (reviewed

in ref. 3). Transcriptional activators and coactivators are responsible for interacting with the preinitiation complex and precisely regulating the rate of transcription initiation in response to developmental and environmental signals (reviewed in ref. 4).

TFIIA was first described as a general initiation factor⁵, and was originally thought to be essential for transcription from many if not all class II nuclear gene promoters. Following mechanistic characterization and cloning of the genes encoding the subunits of TFIIA, however, it is now clear that TFIIA is best defined as a coactivator that supports regulation of pol II transcription (reviewed in ref. 6). Very early in assembly of the preinitiation complex, TFIIA can stably associate with the TFIID-DNA or the TFIIB-TFIID-DNA complexes to give DA or DAB complexes. Both the DA and DAB complexes are considerably more stable than their TFIIA-free counterparts, which allows them to ward off inhibitory coactivators and enhance the stimulatory effects of transcriptional activators.

Providentially, some of these complicated processes can be examined using a simplified system in which TFIID is replaced by TBP. TFIID consists of TBP and more than a dozen tightly associated polypeptides known as TBP-associated factors or TAFs (reviewed in ref. 4). However, recombinant TBP alone can bind both general and regulatory factors, and can direct preinitiation-complex assembly and transcription *in vitro*. TBP has also proved a useful target for X-ray crystallographers. High-resolution structures of apo-TBP and TBP plus DNA (reviewed in ref. 7), and TFIIB recognizing the TBP-DNA complex⁸, have contributed enormously to our understanding of the first two steps of assembly of the preinitiation complex.

With the structure of the TFIIA-TBP-DNA or TA complex in hand, as solved by Tan and co-workers¹ and Geiger and co-workers², we can now model the TAB complex (see figure). TBP recognition of the TATA element proceeds by way of an unprecedented DNA deformation, involving phenylalanine-induced kinking, bending and unwinding. The underside of the molecular saddle is approximated to the widened minor groove face of the TATA element, forming a stable protein-DNA complex. In the TB complex, TFIIB binds to the carboxy-terminal stirrup of the TBP molecular saddle and interacts with the phosphoribose backbone of the promoter both upstream and downstream of the TATA element, recognizing the preformed TBP-DNA complex⁸. TFIIA does much the same with the amino-terminal stirrup of the molecular saddle and the DNA backbone upstream of the TATA element, but on the opposite face of the double helix^{1,2}.



Picture of gene transcription — a model of the complex between transcription factors IIA and IIB, the TATA-box-binding protein (TBP) and the TATA element of DNA. The cocrystal structures of the TB (ref. 8) and TA (refs 1 and 2) ternary complexes were combined to create this hypothetical composite view of the TAB complex assembled on the adenovirus major late promoter. The view is down the approximate intramolecular two-fold axis of symmetry found in TBP, and the transcription start site is labelled +1. The ribbon representations of the proteins are coloured as follows: core TBP's amino-terminal repeat, light blue; core TBP's carboxy-terminal repeat, dark blue; core TFIIB amino-terminal domain, red; core TFIIB carboxy-terminal domain, magenta; TFIIA small subunit, yellow; TFIIA truncated large subunit, green. This model lacks the divergent amino-terminus of TBP, TFIIB's amino-terminal 111 residues, and a non-conserved portion of the large subunit of TFIIA. (Figure courtesy of D. B. Nikolov, prepared with TFIIA-TBP-DNA coordinates supplied by J. H. Geiger and P. B. Sigler.)

How will our model of the TAB complex help us learn more about pol II transcription? First, and most important, the extensive surfaces displayed by the four polypeptide chains in the complex represent presumptive recognition sites for TAFs, pol II and other general initiation factors, and transcriptional activators and coactivators. Systematic programmes of site-directed mutagenesis of the surface of TBP have confirmed the TFIIB and TFIIA binding sites observed in the TB and TA complexes^{9,10}, and demonstrated that the TFIIA binding site is required for activated transcription *in vivo* (A. J. Berk, personal communication).

Second, the structure of the TAB complex underscores the distinct functions of TFIIB and TFIIA. TFIIB interacts with DNA downstream of the TATA element, where it stabilizes the TBP-DNA complex and acts as a precise spacer or bridge between TBP and pol II positioned at the transcription start site

(reviewed in ref. 8). Conversely, TFIIA (which also stabilizes the TBP-DNA complex) is located 3' of the TATA box, where it can be recognized by regulatory proteins that bind to DNA targets upstream of the TATA box and modulate transcription.

Third, the structure of the TAB complex suggests that TFIIA's impact on the biological activity of TFIID (ref. 10) involves the non-conserved amino-terminal portion of TBP and/or the TAFs, because the TBP-DNA complex does not undergo a conformational change when TFIIA binds. Finally, the mechanisms by which TFIIA neutralizes the effects of negative coactivators, which block early steps in preinitiation-complex assembly, are now accessible to directed experimental study. □

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1. Tan, S., Hunziker, Y., Sargent, D. F. & Richmond, T. J. *Nature* **381**, 127-134 (1996).
2. Geiger, J. H., Hahn, S., Lee, S. & Sigler, P. B. *Science* **272**, 830-837 (1996).
3. Roeder, R. G. *Trends biochem. Sci.* **16**, 402-408 (1991).
4. Burley, S. K. & Roeder, R. G. *A. Rev. Biochem.* **65**, 769-799 (1996).
5. Matsui, T., Segall, J., Weil, P. & Roeder, R. G. *J. Biol. Chem.* **255**, 11992-11996 (1980).

6. Zawal, L. & Reinberg, D. *Prog. nucl. Acids Res. molec. Biol.* **44**, 67-108 (1993).
7. Burley, S. K. *Curr. Opin. struct. Biol.* **6**, 69-75 (1996).
8. Nikolov, D. B. *et al. Nature* **377**, 119-128 (1995).
9. Tang, H., Sun, X., Reinberg, D. & Ebright, R. H. *Proc. natn. Acad. Sci. U.S.A.* **93**, 1119-1124 (1996).
10. Lee, D., DeJong, J., Hashimoto, S., Horikoshi, M. & Roeder, R. G. *Molec. cell. Biol.* **12**, 5189-5196 (1992).

A gold mine of methane

William C. Evans

IMAGINE the existence, deeper within the Earth than any organic deposits, of an enormous reservoir of methane (CH_4). Such a reservoir was proposed some years ago by Gold¹, who considered the mantle a possible source of the gas. Sceptics were numerous, but that oft-cited work spurred much interest in finding abiogenic CH_4 from great depth. A suitable candidate was soon found, in the gases dissolved in high-temperature vent fluids on the sea floor at the East Pacific Rise². Although CO_2 is by far the dominant gas in these fluids, several per cent of the total gas³ is CH_4 with an isotopic composition distinctly heavier than that normally produced in the breakdown of organic matter. The source of the CH_4 in these and other similar vents is still debated. In some cases it may be closer to the mantle than many have assumed, as described in a recent paper by Kelley⁴.

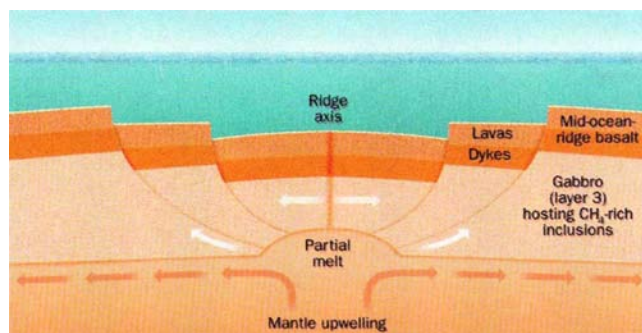
In 1987, a 0.5-km core of oceanic crustal layer 3 was recovered by the Ocean Drilling Program at the Southwest Indian Ridge (SWIR). This layer consists of gabbroic rocks that underlie mid-ocean-ridge basalts (MORB) and extend down several kilometres to the upper mantle. The long core has provided a unique opportunity to study fluid inclusion chemistry in a nearly continuous section of this layer.

Surprisingly, CH_4 is an abundant volatile species in fluid inclusions throughout the length of the core, and in some cases the only major volatile. Estimated pressures of inclusion formation show that this core represents the uppermost part of a 'normal' layer 3 (ref. 5) that had subsequently been unroofed by faulting and then uplifted, making it accessible to drilling. A logical inference is that the whole of layer 3 could be a reservoir of CH_4 -rich fluids. Because this layer constitutes more than half of the oceanic crust, the size of the reservoir is immense, even though the concentration of CH_4 per unit of rock is obviously low. Although commercial extraction of the gas, hosted in tiny unconnected vesicles far below the sea floor, is not a practical procedure, the geochemical implications of such a reservoir are considerable.

Vanko and Stakes⁵ considered the possibility that the CH_4 at SWIR came directly from upwelling mantle, but questioned the exceedingly reducing conditions needed in the mantle to account for fluids so poor in CO_2 . They proposed instead that the transformation of iron- and magnesium-rich upper-mantle rocks into serpentine

minerals during seawater penetration produced the hydrogen needed to reduce seawater carbonates to CH_4 . Kelley has identified⁴ at least two phases of CH_4 production during cooling and alteration of the cored rocks.

In Kelley's view, the first phase of CH_4 production involved magmatic volatiles, trapped within inclusions but continuing to react chemically as the temperature dropped towards 500 °C. Although these inclusions contain mainly CO_2 and water, as expected for magmatic gas, very reduced



Simplified view of the upper 5 km of the spreading centre at the Southwest Indian Ridge. Fluids were trapped within the gabbroic rock as it slowly cooled and moved away from the axis. Subsequent faulting of the crust eventually lifted a large block of the gabbroic layer, containing inclusions rich in CH_4 , to within reach of researchers' drilling.

ing conditions developed, with precipitation of graphite or inward diffusion of H_2 allowing CH_4 concentrations to reach 33 mole per cent in some inclusions.

The second and more pervasive phase took place near 400 °C, as fractures in the cooling rocks began to admit deeply circulating water. The abundant inclusions that were trapped when the fractures later sealed contain CH_4 , water and sometimes H_2 , but no CO_2 . Sea-water-induced serpentinization of iron and magnesium minerals within layer 3 or in the underlying mantle is the favoured production pathway for this CH_4 . This mechanism is bolstered by evidence for present-day CH_4 and H_2 production in ophiolites, by laboratory experiments using sea water with iron and magnesium minerals at high temperature and pressure, and by the CH_4 plumes above serpentinized rock along the Mid-Atlantic Ridge⁶.

Both mechanisms can be examined using isotope analyses of carbon, hydrogen and oxygen. For example, the phase-1 fluid inclusions are a closed system with respect to carbon. Reactions that produce CH_4 from CO_2 concentrate the lighter carbon isotope (^{12}C) in the CH_4 . Conversion of a sizeable fraction of the CO_2 to CH_4 must make the remaining CO_2 heavier than the original mantle gas. Admittedly, the possible formation of graphite is a complication

in the mass balance. The isotopic composition of phase-2 CH_4 is of interest simply because the value expected in this high-temperature *in situ* serpentinization would be difficult to predict. The issue of whether this CH_4 was produced from seawater carbonate should be addressed. If mantle CO_2 was also involved, then layer 3 is an important sink for mantle carbon.

Kelley⁴ also suggests that the fluids trapped in layer 3 may be a previously unrecognized source for volatiles in some hydrothermal sea-floor vents. The fluids are trapped at fairly high temperatures, and so their subsequent release into hydrothermal vent systems is quite plausible. To the extent that they accelerate water-rock interaction, change boiling conditions or mimic biological activities, recognition of these CH_4 -rich fluids is important. Another reason for interest in the fluids is that they could promote transport of those metals whose mobility is increased in reduced states⁷. Such fluids would be most likely to vent at slow spreading centres (like SWIR), or in situations where spreading centres and subduction zones are close (where the downgoing slab remains hot). At axial vents on fast spreading centres, one would expect that the volatiles released during the interaction of sea water with MORB would overwhelm a

layer-3 source, although they might not in Lister's recent model⁷, in which circulating sea water is not heated by magma, but first penetrates hot mantle rocks and then vents through the MORB layer.

Isotopic compositions of volatiles in sea-floor vents are often instrumental in identifying the sources of these species⁸. A thorough isotopic characterization of the SWIR inclusions is needed to test for a layer-3 component in sea-floor vent fluids otherwise thought to be MORB-like. The compositional and isotopic characteristics of MORB volatiles, both in inclusions and vent fluids, are commonly used as well-defined standards in studies of volcanic gases and global geochemical cycles.

Preliminary isotopic analyses⁹ seem to fit the picture described above. Additional work on the isotopes in the drilled core is

1. Gold, T. J. *Pet. Geol.* **1**, 3–19 (1979).
2. Welhan, J. A. & Craig, H. *Geophys. Res. Lett.* **6**, 829–831 (1979).
3. Welhan, J. A. & Craig, H. in *Hydrothermal Processes at Seafloor Spreading Centers* (eds Rona, P. A. et al.) 391–409 (Plenum, New York, 1983).
4. Kelley, J. S. *J. geophys. Res.* **101**, 2943–2962 (1996).
5. Vanko, D. A. & Stakes, D. S. *Proc. Ocean Drill. Program Sci. Results* **118**, 181–215 (1991).
6. Charlou, J.-L. & Donval, J.-P. *J. geophys. Res.* **98**, 9625–9642 (1993).
7. Lister, C. R. B. *Abstr. XXI General Assembly (IUGG) A*, 463 (1995).
8. Lilley, M. D. et al. *Nature* **364**, 45–47 (1993).
9. Kelley, D. S. & Frueh-Green, G. *EOS (Abstr. Fall Meeting)* 675 (1995).
10. Shock, E. L. *Nature* **378**, 338–339 (1995).

underway, and formal publication of detailed results is expected later this year. Although the researchers studying this core have not found a mantle full of methane, they have found out much about the origin of this gas in the deep oceanic crust. Considering that serious uncertain-

ties still exist about the origin of CH_4 in natural gas reservoirs¹⁰, these findings are an important advance. □

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ASTRONOMY

Towards the ideal detector

Francesco Paresce

ASTRONOMERS are uniquely dependent on their ability to wring the most information possible from the usually small number of photons received from the cosmos. Ideally, what one needs is an accurate and reliable determination of the spatial location, the time of arrival, the energy and the polarization of each incoming photon.

In practice, this task is accomplished by a number of more or less complex devices that measure, with varying degrees of success, each of these parameters separately while ignoring the others. It has been a cherished dream of observational astronomers to be able to compress the entire measurement process down to a single element that could perform the operation in one fell swoop. A large step towards this goal has been taken with the achievement, reported on page 135 of this issue¹ by Peacock *et al.*, of single-photon counting with simultaneous energy discrimination, using a niobium-based superconducting tunnel junction (STJ) at visible wavelengths. This development shows the way towards energy- and time-sensitive imaging detectors, for astronomy and for other fields such as medical imaging and remote sensing where these characteristics may be useful.

Energy discrimination by itself is conventionally achieved by using filters to constrain the range of wavelengths reaching the detector, or, for higher spectral resolution, through the use of prisms or diffraction gratings to separate the different wavelengths spatially before detection. The introduction of many extra optical and mechanical elements before the actual detection, however, results in an unavoidable and considerable loss of sensitivity and accuracy.

Recently, imaging spectrometers have been developed that combine optomechanical image slicers and long-slit spectrometers to obtain a true three-dimensional capability² (the two dimensions of the observing plane plus the spectral dimension). They offer, at least in the infrared, considerable improvement in observing efficiency but are still far from ideal. Single-photon counting with imaging capability can be achieved with a number of devices³, but they are all extremely limited in efficiency and speed. All these

methods suffer from the physical separation and inherent inefficiencies of the various measurement processes.

The use of the STJ should relieve, at least in principle, many if not most of these limitations. As an astronomical detector, it follows naturally from the introduction of semiconductor detectors such as charge-coupled devices (CCDs). In such silicon-based devices, the band gap between the ground state and the state excited by the absorption of a photon is comparable to the photon energy. As a consequence, only a single electron is extracted from the detector per absorbed photon, irrespective of the photon energy.

In contrast, the band gap of superconducting niobium as used in the new device is some three orders of magnitude lower, which means that of the order of 1,000 electrons are released per photon. More importantly, although the physics of the STJ detector differs in detail from that of semiconductors (and involves release of charge through the breaking of superconducting Cooper pairs), the average charge generated by an absorbed photon is proportional to its energy. By measuring the charge released, each detected photon can be sorted in energy, to an accuracy limited by the statistical fluctuations in the charge released. The overall quantum efficiency (the proportion of incoming photons detected) is limited only, in principle, by the transmission or reflection properties of the substrate. The STJ efficiency of ~50 per cent compares well with the best CCD devices and is many times higher than any ultraviolet-sensitive device available.

Although the wavelength resolution achieved by Peacock *et al.* ($\Delta\lambda \approx 45$ nm at 350 nm) is modest by conventional spectroscopic standards, this is only the beginning of the development of the STJ as an astronomical detector. Other materials have even lower band gaps than niobium, and therefore promise to yield STJ devices with much higher spectral resolution. Peacock and co-workers are actively pursuing these materials and have already improved on their niobium results with a tantalum-based device. It is unlikely, however, that high spectral resolutions can be achieved soon, so spectroscopy with $\Delta\lambda/\lambda \geq 1,000$ will still have to be performed

using conventional spectrographs. STJs could be envisaged as extremely effective spectral order sorters or used in conjunction with Fabry–Perot-type spectrographs. Their ideal domain of application for now and the foreseeable future is in extremely sensitive continuum observations with moderate resolution.

As one might expect, however, this ability to discriminate photons in energy and arrival time without the use of filters or dispersive elements comes at a price. In order to distinguish the relatively feeble charges generated by the STJ detector above thermal background noise, such devices must operate at extremely low temperatures, typically below 1 K. This requires highly sophisticated cryogenic technology. Moreover, each pixel needs its own amplifier kept at the same temperature, so arrays of many pixels quickly become a topological nightmare to make without producing crosstalk. The device is also very sensitive to acoustic noise and vibrations and to magnetic contamination. Finally, there are count-rate limitations aggravated by sensitivity to low-energy infrared photons, which may demand some kind of filtering that reduces the overall sensitivity of the device. None of these problems is insurmountable, but they will all need very careful attention in order to reduce or eliminate their effects.

Among the many different applications that can be envisaged for this detector, a high priority⁴ is its use in space, in conjunction with a sophisticated ultraviolet to infrared-sensitive telescope with very high spatial resolution and high sensitivity owing to its low background. In particular, it would enable the Hubble Space Telescope to perform spectral-energy-distribution and redshift measurements of the galaxies it can now only detect by broadband imaging. Such an HST-based STJ camera, of perhaps 100 by 100 pixels, may be the only hope on the horizon of accurately measuring the redshifts of most of the 3,000-plus galaxies with visual magnitudes of 27 to 28 that were detected in the Hubble Deep Field.

Even on the ground, these devices will be able, for example, to image a stellar cluster with an 8-m-diameter telescope and in a few minutes obtain not just the conventional colours and magnitudes of all the stars in the field but also their continuum spectra with 10 Å resolution. This promises a true revolution in the way astronomers work in the future. □

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1. Peacock, A. *et al.* *Nature* **381**, 135–137 (1996).

2. Weitzel, L. *et al.* *Astr. Astrophys.* (in the press).

3. Joseph, C. L. *Expl. Astr.* **6**, 97–127 (1995).

4. Newsletter No. 23 (Space Telescope/European Coordinating Facility, Garching, Sept. 1995).

Left, right, left... turn

Rosa Beddington

THE body of every vertebrate has three distinct axes: anterior-posterior, dorsal-ventral and left-right. Of the three, the origin of left-right asymmetry has been the least studied, possibly because it is the last to be determined during embryogenesis, and left-right only acquires meaning once the other two axes are in place.

But we are getting somewhere. Last year the first evidence for left-right asymmetrical gene expression preceding any gross morphological manifestation of the left-right axis was discovered in chick embryos¹. This is now followed by three reports in this issue²⁻⁴ that dramatically demonstrate left-handed gene expression in mammalian and amphibian embryos. What is more, disturbance in the handedness of this gene expression is seen in two mouse mutants in which the left-right axis is partially or completely reversed (a condition known as *situs inversus*); and an unexpected genetic interaction affecting

placement of the organs has been uncovered in embryos doubly heterozygous for targeted mutations in a transcription factor and a secreted protein.

For all that it is the last to be determined, left-right is by no means the least important of the three axes. Left-right asymmetry is essential for normal development and the correct functional juxtapositioning of the organs: heart on the left, liver on the right and so on. Although left and right are not in themselves significant, asymmetry is. *Situs inversus* does not have particularly dire consequences, but ambiguity of the axis does because it affects the interconnection of organs and the partitioning and plumbing of the heart.

What of the new work? On page 151 Meno *et al.*² describe a new divergent member of the transforming growth factor- β (TGF- β) superfamily of secreted molecules, which they call *lefty* because it is expressed exclusively in the mesoderm

of the left lateral plate. This one-sided expression precedes the characteristic right-handed looping of the heart tube and the subsequent axial rotation of the embryo, which normally is towards the right. Remarkably, at a similar stage *lefty* transcripts are also found only in the left half of the floorplate in the developing mid- and hindbrain indicating some hitherto unrecognized asymmetry in this region where axons will cross the midline.

On pages 155 and 158 Collignon *et al.*³ and Lowe *et al.*⁴ show that the murine *nodal* gene, another member of the TGF- β superfamily and one required for mesoderm formation at the onset of gastrulation^{5,6}, is expressed asymmetrically around the node by the early somite stage and its transcripts are only ever found in left-lateral-plate mesoderm, presumably coincidentally expressed with *lefty*. Likewise, a *Xenopus* homologue of *nodal*, *Xnr-1*, is expressed only in left-lateral-plate mesoderm although no comparable asymmetry is apparent around the blastopore.

Although these patterns are intriguing, both the lateness of their one-sided expression and, more importantly, genetic

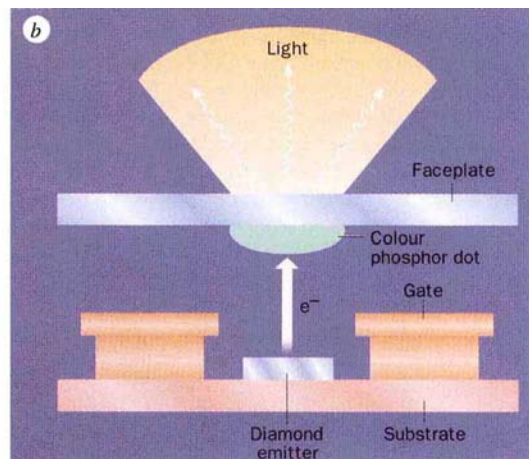
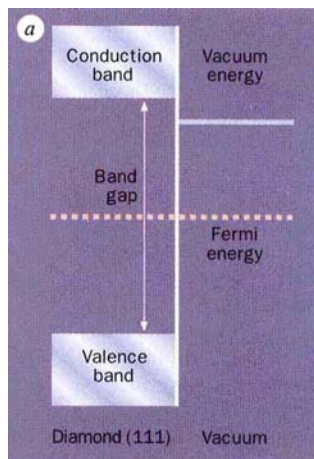
MICROELECTRONICS

Diamond films put on display

VACUUM-TUBE technology is making a comeback, and the report by Ken Okano *et al.* on page 140 will help it on its way. They report efficient electron emission from nitrogen-doped diamond films grown by chemical vapour deposition (CVD), an attribute that augurs well for the use of diamond as a cathode material in diode and field-emission display devices.

Why diamond? It is one of the few materials that possesses a negative electron affinity (NEA), meaning that the bottom of the electronic conduction band lies above the 'vacuum level', the energy of a free electron in a vacuum (part *a* in the figure). So electrons in the conduction band can escape spontaneously from the surface (in fact the electron affinity is surface-dependent: diamond's (111) surface has an NEA, whereas the affinity of the (100) surface is positive). This property gives diamond the potential to act as a 'cold' cathode in vacuum diode devices — conventional vacuum diodes (the old-style 'valves' or 'tubes') relied on thermal activation to achieve electron emission from heated metal filaments.

Cold cathodes can in principle be fabricated on a microelectronic scale, so they could be incorporated into integrated circuits. Here they would offer the potential advantages over solid-state devices of high speed, energy efficiency and resistance to radiation damage — all a consequence of the



fact that the carriers do not move through the solid state but through a vacuum. In addition, cold cathodes could provide electron emitters for phosphorescent display devices (part *b* in the figure), whose fabrication requires only simple lithographic procedures. Several devices of this kind have now been demonstrated.

In pure diamond, the conduction band is empty — the material is an insulator. So it must be doped (either p-type or n-type) to provide mobile charge carriers. To a first approximation, the greater the degree of doping, the lower the threshold voltage needed to attain efficient emission and high current densities. Doping with boron gives only a small emission enhancement, phospho-

rus doping does better, and nitrogen doping appears best of all (M. W. Geis *et al.*, *Appl. Phys. Lett.* 67, 1328; 1995). But Murphy's law applies: getting nitrogen into CVD diamond films in high concentrations is hard.

This is where Okano *et al.* have taken the crucial step. They find that using urea as the nitrogen source in the CVD synthesis gives films with a high nitrogen content — around 0.2 per cent. The diamond cathodes so fabricated have threshold voltages low enough that adequate emission currents for display and microelectronic applications should be possible in vacuum devices powered by a single commercial 1.5-volt battery.

Philip Ball

analysis suggest that both *nodal* and *lefty* are downstream of left-right determinants. Two mutations are known that alter situs in mice: *inv*⁷, which was caused by a transgene insertion and completely reverses the left-right axis in almost all homozygous embryos; and *iv*⁸, which randomizes the left-right axis so that about 50 per cent of homozygotes exhibit *situs inversus*. In *inv/inv* embryos both *lefty* and *nodal* are expressed only on the right side; and in *iv/iv* embryos, as befits the 'random' phenotype of the mutation, *nodal* expression is either normal, absent or switched to the right, and *lefty* too is inverted in half the homozygotes.

So both of these signalling molecules may be involved in the execution of left-right asymmetry, possibly by effecting differential growth such that both the heart tube and the embryo turn away from where these molecules are being produced. But neither can be instrumental in establishing left-right asymmetry.

Are there any other clues to explain the sinistral expression of *lefty* and *nodal*? The *nodal* expression pattern corresponds to that of the chick *Nodal-Related 1* gene whose left-handed expression has been shown to be dependent on preceding asymmetrical expression of *sonic hedgehog* (*shh*)¹. However, Collignon *et al.*³ were unable to detect any asymmetry in the distribution of *shh* transcripts around the mouse node, although it remains possible that differential proteolytic processing of the protein⁹ could generate functional asymmetry.

In chick embryos the asymmetrical expression of *shh* seems to depend on another TGF- β signalling system. More *activin receptor IIa* transcripts are found to the right of the node but they can be increased experimentally on the left by introducing an activin-coated bead on the left side. This seems to abolish the asymmetry of *shh* expression, and instead of the heart always looping to the right it loops randomly with respect to left and right¹. However, in the mouse, mutations which remove all embryonic activins and inhibin¹⁰ or activin receptor II (ref. 11) do not perturb the left-right axis. So although the *nodal* profile seems to be the same in chick and mouse, there appear to be discrepancies with respect to the putative upstream components of the pathway.

As a further twist to the story, Collignon *et al.*³ have found an intriguing genetic interaction in embryos which are double-heterozygotes for mutations in *nodal* and *HNF3- β* , a member of the *fork-head* gene family of transcription factors and another prominent and highly conserved player in embryonic patterning. Such double-heterozygotes express *nodal* in the lateral mesoderm on both sides, and 70 per cent of these embryos later display misplaced abdominal viscera and cardiac abnormalities. This implies that protein levels, as predicted by gradient and threshold models¹², are crucial for the correct handedness of the embryo. In the chick, *HNF3- β* is expressed very transiently on the left side of the streak immediately behind the node and this is ascribed to its local induction by *Shh*¹.

Therefore, despite its symmetrical expression pattern in the mouse, a role for *shh* is again invoked by the interaction between *nodal* and *HNF3- β* . Whether or not *shh* really is involved will only be proved if *shh*⁻/*shh*⁻ embryos exhibit *situs* defects and these have yet to be reported. Meanwhile, identification of the disrupted genes in *iv* and *inv* mutations, which do not correspond to any of the known molecular players identified so far, remains an essential step for understanding mammalian left-right asymmetry and almost certainly for unravelling the pathway in all other vertebrates. □

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VISUAL PSYCHOPHYSICS

Only one speed per object

Oliver Braddick

VISUAL psychophysics is the attempt to tease out the organization of the visual system by quantitatively examining the sensitivity and precision of human judgements. Typically, subjects are required to detect the presence of a faint signal, or match simple qualities such as the speed of two moving patterns. Such experiments may appear to be focused on rather narrowly defined judgements, but they are entangled with deep and difficult questions of how our subjective experiences reflect the pattern of activity in brain pathways.

Traditionally, psychophysical experiments of this kind have aimed to reduce the test stimulus to its simplest form, and to purify the observer's task of any complications that might arise from 'higher level' processes of global perceptual organization and interpretation. Such stripped-down judgements were intended to characterize the basic neural mechanisms that encode properties such as colour, contour orientation and motion early in the brain's visual processing.

Work reported by Verghese and Stone on page 161 of this issue¹ exemplifies a movement that is breaking down this isolationism of visual psychophysics from the broader perceptual process. There has been a surge of experiments showing that 'low level' judgements such as colour matching, direction of motion and texture detection can be strongly affected by the perceived organization of a scene into occluding and occluded surfaces^{2,3}, the focus of attention⁴ or past experience in a related judgement⁵. In a parallel development, visual neuroscientists have moved beyond manipulations of the simplest properties of stimuli and have shown the influence of attention (for example ref. 6)

and perceptual organization⁷ on single-neuron activity in visual areas of the primate cerebral cortex.

A key process in perceptual organization is the structuring of the visual field into distinct objects: two neighbouring regions may be processed quite differently depending on whether they are grouped into a single visual object, or segmented between two. In this¹ and a previous paper⁸, Verghese and Stone show that the grouping process can determine a simple psychophysical judgement of pattern speed.

Last year⁸, they reported that speed judgements were not improved by a six-fold increase in the area of a single patch of moving pattern, but that precision could be doubled if the motion appeared in six separate patches. They argued that each segmented patch provides a single estimate of speed, and that combining independent estimates from several patches reduces random error. Their new experiments¹ strengthen the argument that segmentation is the critical variable. First, sensitivity is improved by splitting a single banana-shaped patch of moving pattern into three separate circular patches, despite minimal change to area or to position in the visual field. Second, the similar improvement from dividing a circular region into four quadrants is nullified if this division appears to result from a superimposed cross that partly occludes a unitary region. In this experiment perceptual organization produces a change in sensitivity despite identical geometry of the quadrants in the two cases.

The addition of segmentation to the list of perceptual processes that can affect simple visual judgements is interesting, but perhaps not surprising. The deeper

1. Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. & Tabin, C. *Cell* **82**, 803-814 (1995).
2. Meno, C. *et al.* *Nature* **381**, 151-155 (1996).
3. Collignon, J., Varlet, I. & Robertson, E. *J. Nature* **381**, 155-158 (1996).
4. Lowe, L. A. *et al.* *Nature* **381**, 158-161 (1996).
5. Conlon, F. L., Barth, K. S. & Robertson, E. *J. Development* **111**, 969-981 (1991).
6. Zhou, X. *et al.* *Nature* **361**, 543-547 (1993).
7. Yokoyama, T. *et al.* *Science* **260**, 679-682 (1993).
8. Layton, W. J. *J. Hered.* **67**, 336-338 (1976).
9. Porter, J. A. *et al.* *Nature* **374**, 363-366 (1995).
10. Matzuk, M. M. *et al.* *Nature* **374**, 354-356 (1995).
11. Matzuk, M. M., Kumar, T. R. & Bradley, A. *Nature* **374**, 356-359 (1995).
12. Brown, N. A. & Wolpert, L. *Development* **109**, 1-9 (1990).

significance of this work lies in the nature of the effect, and the questions it raises about how neural signals underlie perceptual judgements. This issue has, so far, been explored furthest for the judgement of detecting very faint signals. Notably, Britten, Newsome and colleagues^{9,10} have measured the response of single cells in the motion-responsive area MT (V5) of monkey cortex, using stimuli in which a few coherently moving dots are embedded in randomly moving noise. Individual cells have a statistically reliable response at noise levels very close to that at which the trained monkeys can just respond to the motion. Detailed analysis¹⁰ suggests that the animals (and presumably human observers, whose threshold is very similar) are combining information from a small group of neurons (say 30–50) with similar properties.

The human threshold for detecting coherent motion in noise improves with increasing area of the patch of moving dots, up to 25 square degrees of visual angle¹¹. It's not yet clear how far this results from using the full receptive field area of the relevant MT neurons, and how far it reflects an increase in the pool of neurons from which information is combined. However, it is striking that the ability to detect motion increases over this range of area, while the ability to judge the speed of motion does not. To explain detection performance, we invoke the benefit of combining signals from several neurons, which tends to cancel out the effects of random noise. The large patch in Verghese and Stone's speed-judgement experiment must also have activated many MT neurons; yet the benefits of combining independent estimates only appeared when each estimate could be obtained from a distinct patch. Clearly, the way neural signals can be used in detection at threshold must be rather different from the way they are used in estimating a parameter such as the speed of a readily detectable (suprathreshold) motion.

So far we can only speculate what the basis of this difference might be. One possibility is that it depends on the nature of the information required. Neural models of visual parameter estimation (for colour, disparity or orientation, as well as for speed) almost all suppose that signals must be compared from two or more neurons with different sensitivity functions for the parameter in question. Perhaps the need for this type of interaction among a set of neurons with different properties precludes the combination of information from spatially distributed neurons that have similar properties. On this view, within each segmented visual object only one instance of the comparison process deriving speed could operate, to yield a single parameter for that object. An intriguing analogy may be provided by the recent report¹² that accurate judgement of

grating orientation requires focal attention, whereas detection of a faint grating does not.

On an alternative view, the difference may be a consequence of effects which come into play only with suprathreshold stimuli. The advantage of combining signals from many neurons occurs only if the random noise is at least partly uncorrelated between neurons. There is evidence that when cortical neurons are responding to a common stimulus, their patterns of impulses show a close temporal correlation. It has been suggested that this synchronization depends on the visual elements that activate each neuron being grouped as a common visual object¹³. It is possible, then, that when the regions within a single visual object activate a set of neurons sufficiently strongly, the activity of the neurons becomes entrained together (synchronized) — indeed, this may be the neural signature of perceptual grouping. Such entrainment, however, would abolish the degree of independence that the impulse patterns show when they are responding to weak stimuli near threshold, meaning that in suprathreshold conditions the firing of many sets of neurons carries no extra information for parameter estimation beyond that provided by a single set.

It should be possible to test these speculations psychophysically (and perhaps neurophysiologically too). We need data on whether segmentation affects the way information is combined in detecting motion coherence, and it should be possible to make detection and speed discrimination judgements with the same near-threshold stimuli, even at the same time. As well as the intrinsic importance of discovering how perception depends on neural activity, deeper understanding of this relation is the key to better use of psychophysical data for revealing human visual function at the single-neuron level. □

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1. Verghese, P. & Stone, L. S. *Nature* **381**, 161–163 (1996).
2. Braddick, O. J. *Nature* **333**, 803–804 (1988).
3. Nakayama, K., Shimojo, S. & Ramachandran, V. S. *Perception* **19**, 419–560 (1990).
4. Chaudhuri, A. *Nature* **344**, 60–62 (1990).
5. Karni, A. & Sagli, D. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4966–4970 (1991).
6. Moran, J. & Desimone, P. *Science* **229**, 782–784 (1985).
7. von der Heydt, R. & Peterhans, E. *J. Neurosci.* **9**, 1731–1748 (1988).
8. Verghese, P. & Stone, L. S. *Vision Res.* **35**, 2811–2823 (1995).
9. Newsome, W. T., Britten, K. H. & Movshon, J. A. *Nature* **341**, 52–54 (1989).
10. Britten, K. H., Shadlen, M. N., Newsome, W. T. & Movshon, J. A. *J. Neurosci.* **12**, 4745–4765 (1992).
11. Downing, C. J. & Movshon, J. A. *Invest. Ophthalm. vis. Sci. (Suppl.)* **30**, 72 (1989).
12. Wen, J., Koch, C. & Braun, J. *Invest. Ophthalm. vis. Sci. (Suppl.)* **37**, S532 (1996).
13. Engel, A. K., Koenig, P., Kreiter, A. K., Schillen, T. B. & Singer, W. *Trends Neurosci.* **15**, 218–226 (1992).

Grave solutions

MAD cow disease is still headline news. A molecule of the cow's brain protein gets wrongly folded into a 'prion'. This somehow persuades other protein molecules to fold the wrong way too. Prionic protein spreads relentlessly through the creature's brain.

Brains seem strangely vulnerable to misfolding proteins. Even Alzheimer's disease seems to develop by the spreading of amyloid plaques of knotted protein. What we need, says Daedalus, is some sort of 'molecular penetrating oil' that will insinuate itself into protein molecules and force them to unwind. The oil he proposes is heavy water.

Most proteins are copiously hydrated. Each folded molecule is stitched together by hydrogen bonds, into a shape defined by a subtle and delicate balance of bond energies. Replace hydrogen by deuterium, and all these will change. The deuterium bonds will be slightly longer and weaker. The protein will be more open, and less rigidly folded.

Sadly, you can't cure a mad cow or Alzheimer's victim with heavy water. The stuff is poisonous in large doses, as if it altered protein folding too strongly. But Daedalus reckons that heavy water is only toxic by conflict with light water. A partly deuterated organism, he says, is racked by deadly inconsistencies and imbalances. He plans to take the body in one stride from full hydrogenation to full deuteration, without lingering in the dangerous mixed region between.

His first idea was to freeze-dry the patient to a desiccated shell, and then rehydrate him with heavy water. But he now favours a less extreme procedure. The patient will be made desperately thirsty, and then anaesthetized (possibly with heavy alcohol). He will be placed in a cold bath of heavy water, to chill his metabolism almost to a halt while soaking into his skin. Simultaneously, a massive transfusion will replace his blood with previously prepared heavy blood. On being warmed up again, he will wake up a fully deuterated 'heavy man', little damaged by the swift exchange. His prion diseases, not to mention facial wrinkles, hardened arteries, arthritic joints, long sight and other consequences of ancient, gnarled protein molecules, should soon unwind. When they have, he could be lightened again.

Daedalus will perfect the technique by animal trials: first with jellyfish (which should deuterate easily), then mice, monkeys and human volunteers. The final process, though expensive, could transform our health, mental vigour and longevity. Once the human population had been treated, it could then be tried on all those mad cows.

David Jones

Prediction of future BSE spread

SIR—Spongiform encephalopathies are progressive diseases caused by unconventional infectious agents, now generally believed to be malformed prion proteins¹⁻³. At present, there is no test for infection: there is generally a long incubation period between infection and onset of symptoms, and the condition can only be confirmed after death. In 1986 a new form of this disease, bovine spongiform encephalopathy (BSE), was identified in cattle in the United Kingdom⁴. The origin of this disease is believed to be food containing the remains of sheep, and later cattle, that were infected with either scrapie or the BSE agent. In July 1988, the UK government banned the addition of material derived from ruminants to cattle feed. To date, about 160,000 cases of BSE have been reported in the United Kingdom, out of which at least 28,000 occurred in animals born after the food ban; there has been a continued use of contaminated food⁵⁻⁷. Many more animals are likely to have been infected but were slaughtered before symptoms developed.

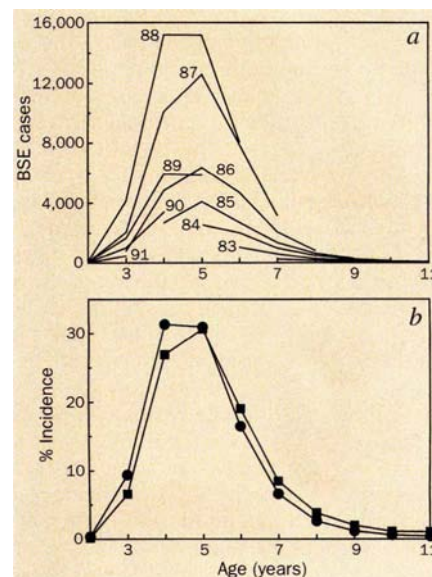
Extensive data are now available on the number of BSE cases in different age cohorts of cattle in the past 5 years⁸ (we are most grateful to J. Wilesmith, J. Ryan, L. Hoinville and their colleagues at the Central Veterinary Laboratory for their generous help with data), and we report here results of the simplest of the initial analyses on the dynamics of the disease. Making certain assumptions, we can determine if there have been changes in the age pattern of cases and can predict the number of future cases in different age classes. The figure (a) shows the number of BSE cases in different age cohorts (animals born in a given year). The shapes of these curves are very similar, with a peak between 4 and 6 years of age.

The data provide information on $x_i(t)$, the number of BSE cases with a clinical

onset in year t in animals aged i years. Here, i goes from 2 to 11 years and t from 1989 to 1994. The figures for 1995 are not yet final, because there are still a few cases being reported with an onset in 1995. Most animals are infected within the first year of life, when the use of feed containing ruminant protein is high⁵. If there is a non-changing, age-dependent frequency of expression of disease, then $x_i(t)$ can be written as $x_i(t) = I(t-i)P_i$, where $I(t-i)$ is the number of animals infected in year $t-i$ and P_i is the probability that an animal infected in its first year of life exhibits the disease at age i . We can then estimate the relative incidence rate, P_{i+1}/P_i , from the quantities $r_i(t+1) = x_{i+1}(t+1)/x_i(t)$, and make predictions for subsequent years. There is, however, a slight trend for $r_i(t)$ to decrease with t , which indicates a small shift of the incubation period to disease expression at a younger age (b in the figure).

Therefore, we present two types of predictions. For the first prediction we use the average $\bar{r}_i = \sum_t x_{i+1}(t+1)/\sum_t x_i(t)$ as estimators. For the second model we use $\bar{r}_i = r_i(1994)$ as the most recent estimators. In both cases, we then predict $x_{i+1}(1995) = \bar{r}_i x_i(1994)$, and similarly each subsequent year using the prediction for the previous year. Because $r_i(t)$ values are decreasing slowly with time, the second model gives a lower estimate; if this trend reflects a change in the underlying dynamics during the course of the epidemic, the second estimate should be more accurate.

In the table we give, by age cohort, the number of cattle predicted to develop BSE over the next 5 years. A comparison of the predicted number of cases for 1995 with the as yet incomplete data for 1995 gives close agreement: 13,267 confirmed cases, which are likely to constitute at least 90% of the total cases, compared with 13,880–16,110 predicted. From 1996 until 1999, we expect between 15,000 and



a, Numbers of BSE cases in different age cohorts, labelled by year of birth, indicative of common underlying incubation distribution. b, Estimates of the age-dependent frequency of disease expression, including background mortality. Squares, the estimate using an average over all the data; circles use only the data from 1993 and 1994. Both estimates give peak incidence of disease at 4 and 5 years of age. There appears to be a slight shift towards expression of the disease in younger cattle in later years.

24,000 cases of BSE to arise in animals born before 1993. This prediction is representative of the total number only if infection of new animals finally ceases.

On the basis of our predictions and the knowledge of the age distribution of adult animals in UK cattle herds, we can calculate that the highest incidence per capita will be in those animals born between 1987 and 1990, with a marked peak in 1988, the year in which the ban on animal supplements in feed was introduced. The lowest per capita and absolute incidence will be in cattle currently aged 10 or more years, as the age-dependent frequency of disease expression is past its peak. About 75% of future cases will occur in animals born in 1989 or later (at least 5 months after the feed ban); this reflects the mid-age peak of the age distribution for the expression of the disease (see figure) and the continuing, though diminishing, rate of infection⁵.

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1. Prusiner, S. B. et al. (eds) *Prion Diseases of Humans and Animals* (Ellis Horwood, Chichester, 1992).
2. Weissmann, C. *Nature* **352**, 679–683 (1991).
3. Prusiner, S. B. *Science* **272**(1), 48–57 (1995).
4. Wells, G. A. H. et al. *Vet. Rec.* **121**, 419–420 (1987).
5. Wilesmith, J. W. & Ryan, J. M. B. *Vet. Rec.* **132**, 300–301 (1993).
6. Hoinville, L. J. et al. *Vet. Rec.* **136**, 312–318 (1995).
7. Wilesmith, J. W. et al. *Res. Vet. Sci.* **52**, 325–331 (1992).
8. MAFF *Prog. Reps* (1989–96).

PREDICTION OF THE NUMBERS OF BSE CASES BY AGE FOR THE YEARS 1995–99					
Age	1995	1996	1997	1998	1999
3	330–490 (435)				
4	1,520–1,860 (1,660)	1,100–2,000			
5	3,360–3,870 (3,221)	1,510–2,120	1,090–2,280		
6	3,120–3,650 (2,942)	1,790–2,410	800–1,320	580–1,420	
7	3,260–3,620 (2,957)	1,260–1,630	720–1,080	320–590	230–640
8	1,260–1,420 (1,021)	1,290–1,620	500–730	290–480	130–260
9	370–440 (311)	570–760	590–860	230–390	130–260
10	150–160 (117)	190–240	290–420	300–480	110–210
11+	90–120 (67)	100–150	130–230	200–390	210–450
Unknown	420–490 (523)	250–340	130–220	60–120	30–60
Total	13,880–16,110 (13,267)	8,050–11,270	4,250–7,130	1,970–3,870	840–1,880

In each column, the lower estimate is based only on the data from 1993 and 1994, and the higher estimate uses an average over all the data. The few cases of unknown age are estimated at 3% of the total number of cases, as has occurred in the years 1989 to 1994. The numbers in parentheses are the 1995 cases reported to date, thought to be at least 90% of the 1995 cases. Using the lower estimate, we predict ~11,000 further cases between 1996 and 1999 in animals born after the feed ban.

Even smaller radar tags on insects

SIR—Movement behaviour is fundamental to the understanding of insect population dynamics¹. Unlike researchers working on vertebrate species, insect ecologists have not had the benefit of such technology as radio-collaring for tracking animal movements. Walking beetles have been fitted with 'tags' that reflect harmonic radar²; these tags have now been reduced in size so that bees can carry them and be detected using powerful radar signals³. To ensure that the tag attached to the insects does not itself alter their normal movement, its size and weight must be extremely small. Here we report extreme miniaturization of such a tag to an order of magnitude smaller than that previously used³, and describe its use with portable radar equipment in the field on a variety of insect species (Fig. 1).

The tag (Fig. 2) is glued to the insect thorax or abdomen with rubber cement, and the near-invisible dipole simply trails behind the insect. This configuration is two orders of magnitude lighter than that used for tracking ground beetles², and an order of magnitude lighter than that used for tracking bees³. Our transmitter-receiver (RECCO Rescue Systems, Lidingö, Sweden) is carried in a portable backpack, weighs 8 kg and transmits a 1.7-W continuous microwave frequency of 917 MHz from a hand-held Yagi antenna. The tag on the target insect reflects this energy at a frequency of 1,834 MHz, which is detected by the same hand-held antenna. The use of a longer frequency than that used for tracking bees³ reduces the attenuation of the signal by water in intervening vegetation. The current configuration of transmitter-receiver and dipole permits detection at up to 50 m. Tagged insects are relocated at intervals by systematic searching and triangulation on the reflected signal.

We have successfully fitted tags to five species of flying insects with a range of body weights: the small Apollo butterfly *Parnassius sminthius* (350 mg), the common alpine butterfly *Erebia epipsodea* (150 mg), both the larvae and moths of the forest tent caterpillar *Malacosoma disstria* (200 mg; Fig. 2a), and the parasitic tachinid fly *Patelloa pachypygus* (45–60 mg; Fig. 2b) and sarcophagid fly *Arachnidomyia aldrichi* (55–75 mg), both of which attack the tent caterpillar. The tag increases the weight of these insects by only 0.1–0.9%. Tagged insects can be used to examine the effect of habitat structure on insect movement. Over a period of 3 hours, we repeatedly located tagged parasitic

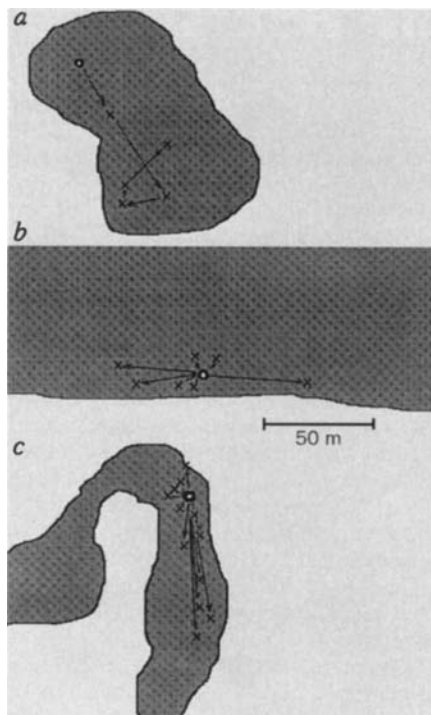


FIG. 1 Relocations and trajectories from a release point for: a, a single *Patelloa pachypygus* fly over a 3-h period; b, c, forest tent caterpillar moths over 48 h, all within forest stands of trembling aspen (shaded) adjacent to clearings (unshaded).

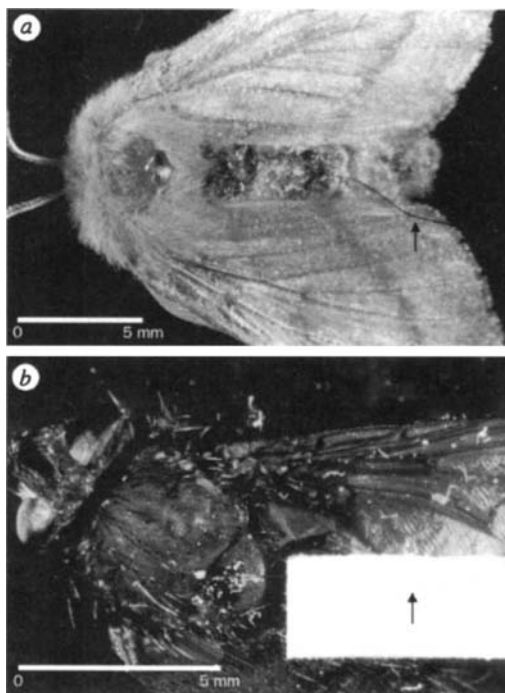


FIG. 2 Forest tent caterpillar moth (a) and tachinid fly *Patelloa pachypygus* (b) fitted with tags made from a Schottky low-barrier diode (Hewlett-Packard, HP 5082-2837) mounted in the centre of an 8-cm dipole antenna of extremely fine aluminium bonding wire (arrows). Total weight of this configuration is 0.4 mg.

flies moving within a forest stand (Fig. 1a); after 48 hours, the moth stage of their tent caterpillar hosts was relocated up to 100 m from the point of release (Fig. 1b). In a mark-recapture study of the Apollo butterfly, we repeatedly recaptured 10 individuals fitted with tags, together with untagged butterflies, over a period of 2 weeks. Butterflies did not seem to be affected by the 0.4-mg tags.

Movement data can be collected by one or more researchers, each carrying a transmitter-receiver. Use of tags small enough so as not to alter insect movement, in combination with more powerful transmitters, may provide a tool for studying movement, the 'missing element' of insect population dynamics.

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1. Turchin, P. *Ecology* **72**, 1253–1266 (1991).

2. Mascanzoni, D. & Wallin, H. *Ecol. Ent.* **11**, 387–390 (1986).

3. Riley, J. R. *et al. Nature* **379**, 29–30 (1996).

Reducing antibiotic resistance

SIR—A reduction in the use of antibiotics has recently been proposed as a measure to forestall, and ideally reverse, the growing public-health problem of antibiotic-resistant bacteria^{1–3}. This recommendation implicitly assumes that resistance to antibiotics reduces the fitness of bacteria in the absence of antibiotic-mediated selection, thereby allowing sensitive bacteria to replace resistant strains in drug-free environments. We now show that, although mutations to antibiotic resistance in bacteria may initially impose a considerable fitness burden, natural selection can result in the rapid fixation of second-site, compensatory mutations which substantially reduce this cost by restoring physiological functions impaired by resistance mutations without altering the level of bacterial resistance.

Some point mutations in the *rpsL* gene in the bacterium *Escherichia coli* confer resistance to high concentrations of streptomycin, but reduce the darwinian fitness of bacteria by decreasing peptide chain elongation rates^{4,5}. We considered two spontaneous streptomycin-resistant (*Str*^r), *rpsL* mutants of *E. coli* CAB281 (see table). One mutant had a 14% per generation disadvantage relative to the *Str*^r parental strain, and the other had a 19%

ADAPTATIONS TO FITNESS COSTS OF STREPTOMYCIN RESISTANCE

Strain*	Evolutionary status	DNA sequence (amino acid) at codon 42†	Cost of resistance (% per generation, ± 1 s.e.)‡	Peptide chain elongation rate (amino acid per s, ± 1 s.e.)§
CAB281	Baseline	AAA (Lys)	—	18.26 $\pm$ 1.41
CAB281-1	Evolved	AAA (Lys)	—	20.48 $\pm$ 2.10
STR1	Baseline	ACA (Thr)	13.6 $\pm$ 0.57	10.60 $\pm$ 0.70
STR1-1	Evolved	ACA (Thr)	6.97 $\pm$ 0.60	17.54 $\pm$ 1.10
STR1-2	Evolved	ACA (Thr)	4.63 $\pm$ 0.55	17.93 $\pm$ 1.60
STR2	Baseline	AAC (Asn)	18.8 $\pm$ 0.79	8.74 $\pm$ 0.56
STR2-1	Evolved	AAC (Asn)	9.55 $\pm$ 1.3	18.45 $\pm$ 1.59
STR2-2	Evolved	AAC (Asn)	6.68 $\pm$ 0.51	18.47 $\pm$ 1.77

*CAB281, donated by C. A. Bloch, is an *E. coli* K1/K12 chimera in which the K1 *kpsA* cluster has been lost. We selected spontaneous streptomycin-resistant mutants from a CAB281 population grown in the presence of 100 $\mu\text{g ml}^{-1}$ streptomycin. We froze all strains in 15% glycerol immediately after isolation to prevent further genetic changes.

†We isolated chromosomal DNA from each strain and amplified it by PCR using primers external to the coding sequence of the *rpsL* gene. We sequenced both strands of this DNA fragment, either by double-strand cycle sequencing using external and internal primers, or by automated sequencing by the Molecular Genetics Instrumentation Facility of the University of Georgia (Athens) using the same external primers. We found strain differences only at codon 42.

‡Estimated by direct competition against an initially equal density of wild type¹⁰.

§Estimated by measuring the time needed to synthesize a complete molecule of β -galactosidase, according to refs 4, 11.

per generation disadvantage, corresponding to different single-base substitutions in codon 42 of the *rpsL* gene (see table). The rates of peptide chain elongation of both mutants were also significantly lower than that of the Str^s parental strain.

To examine how bacteria adapt to the fitness costs associated with these *rpsL* mutations, we established 10 replicate, long-term serial transfer cultures⁶ for each class of Str^r mutant in antibiotic-free minimal medium. All cultures were maintained for 135 generations. Because evolution occurred in the absence of antibiotics, higher-fitness Str^s wild-type revertants could have dominated these cultures. On the contrary, however, we found that of 200 randomly chosen colonies tested from each of the two series of 10, 135-generation cultures, all were resistant to high concentrations of streptomycin.

We randomly chose two 135-generation cultures of each Str^r mutant class, allowed these four cultures to evolve for an additional 45 generations of culture, and isolated one, presumably evolved, colony from each of these 180-generation cultures. The *rpsL* gene sequence in all four 180-

generation strains is the same as the baseline sequence (see table). The cost of resistance in all four strains, estimated by direct competition experiments against the wild-type Str^s strain, is significantly lower than costs in their ancestors (table). One evolved mutant (strain STR2-1), however, has a higher cost of resistance than the other evolved strains, suggesting that compensation for the cost of resistance may have occurred by more than one mechanism.

The peptide chain elongation rates of all four evolved, higher-fitness Str^r mutants are significantly greater than those of the unevolved, parental Str^r strains, and not significantly different from that of the Str^s wild-type strain (table). To see whether increased elongation rates resulted simply from a general adaptation of the CAB281 genetic background to experimental culture conditions, we maintained two independent cultures of wild-type CAB281 for 180 generations. We then screened 30 single colonies from each evolved CAB281 culture for fitness improvements, by direct pairwise competition against the wild-type strain with an added neutral Nal^r marker. The clone (strain CAB281-1) with the largest fitness improvement, 6% relative to wild type, shows no significant increase in elongation rate relative to that of the original wild-type strain.

Thus, in the absence of streptomycin, the initially high cost of chromosomal resistance to this antibiotic is rapidly compensated for without a clinically significant reduction in the level of streptomycin resistance. This compensation is achieved by second-site, non-*rpsL* mutations in loci yet to be determined, and in all cases is associated with a return to wild-type (or near wild-type) rates of peptide chain elongation.

As well as demonstrating the physiol-

ogical basis of a rapid adaptation, the present results, together with evidence that similar compensation may be possible for the fitness costs of plasmid carriage⁷⁻⁹, suggest that a reduced incidence of antibiotics may not lead to decreases in the current frequency of resistant bacteria. We are not arguing against prudent antibiotic use, as the frequency of bacteria resistant to antimicrobial agents is proportional to the extent to which these agents are used for therapy and prophylaxis². On the other hand, our results are a warning that prudence alone may not be sufficient to stem the tide of antibiotic resistance.

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Obligate anaerobe or not?

SIR — Silberman *et al.*¹ in Scientific Correspondence state that *Blastocystis hominis*, a parasite of the human intestine, is an obligate anaerobe. Many lower eukaryotes that inhabit regions where the O₂ supply is limited or episodic not only are capable of withstanding long exposure to low concentrations of O₂, but may even use it for energy generation, or in some cases even require it for growth and division².

At the mucosal lining of the upper intestine³, O₂ reaches 60 μM , and another protozoal parasite, *Giardia lamblia*, has magnificently tuned mechanisms for adjusting its intracellular redox states and fermentative fluxes to accommodate this⁴. Even in the vagina, where less O₂ is available, *Trichomonas vaginalis*, another amitochondriate flagellate, benefits from O₂-enhanced electron transport⁵. Controlled culture conditions confirm higher growth rates and yields, and make *T. vaginalis* a microaerophile rather than merely an aerotolerant anaerobe.

It would be surprising if *B. hominis*, replete with mitochondria, is really an obligate anaerobe; as for so many 'anaerobic' metazoans, we do not yet know enough to be sure that O₂ is not essential in its metabolism or life cycle.

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- Office of Technology Assessment *Impacts of Antibiotic-Resistant Bacteria* (OTA, US Congress, Washington DC, 1995).
- WHO Scientific Working Group on Monitoring and Management of Bacterial Resistance of Antimicrobial Agents (World Health Organization, Geneva, 1995).
- Levy, S. B. *Trends Microbiol.* **2**, 341-343 (1994).
- Zengel, J. M., Young, R., Dennis, R. R. & Nomura, M. *J. Bact.* **129**, 1320-1329 (1977).
- Bilgin, N., Claessens, F., Pahverk, H. & Ehrenberg, M. *J. molec. Biol.* **224**, 1011-1027 (1992).
- Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. *Am. Nat.* **133**, 1315-1341 (1991).
- Bouma, J. E. & Lenski, R. E. *Nature* **335**, 351-352 (1988).
- Modi, R. I. & Adams, J. *Evolution* **45**, 656-667 (1991).
- Lenski, R. E., Simpson, S. C. & Nguyen, T. T. *J. Bact.* **176**, 3140-3147 (1994).
- Lenski, R. E. in *Assessing Ecological Risks of Biotechnology* (ed. Ginzburg, L. R.) 173-192 (Butterworth-Heinemann, Boston, 1991).
- Dalbow, D. G. & Young, R. *Biochem. J.* **150**, 13-20 (1975).

- Silberman, J. D., Sogin, M. L., Leiper, D. D. & Clark, C. G. *Nature* **380**, 398 (1996).
- Williams, A. G. & Lloyd, D. *Adv. microb. Ecol.* **13**, 211-262 (1993).
- Atkinson, H. in *Nematodes as Biological Models* Vol. 2 (ed. Zuckerman, B. H.) 101-131 (Academic, London, 1987).
- Paquet, T., Kelly, M., Jarrold, E. L., Lindmark, D. G. & Lloyd, D. *Molec. biochem. Parasit.* **57**, 65-72 (1993).
- Paquet, T. & Lloyd, D. *Molec. biochem. Parasit.* **41**, 65-72 (1990).
- Bryant, C. (ed.) *Metazoan Life Without Oxygen* (Chapman & Hall, London, 1991).

Sinistral nematode population

SIR — Several animal taxa display a consistent left–right asymmetry of the body plan. In nematodes, dextrality predominates¹. However, we have now found a nematode species that has sinistral populations.

In nematodes of the order Rhabditida, the anterior gonadal arm generally lies on the right of the intestine. Together with other asymmetries at the cellular level found in other nematode orders^{1,2}, this defines the overall body handedness. After looking at many wild isolates of free-living soil nematodes, we found one population (strain PS1158) with reversed handedness. We identified it as *Acroboloides bodenheimeri* Steiner, 1936³, Family Cephalobidae, Order Rhabditida. Type specimens for this species and for two independent isolates are also sinistral (see table). Other fixed and cultured isolates, previously identified by morphological criteria as *A. bodenheimeri*⁴, are dextral (for example, strain PS2052). PS2052 crosses with neither PS1158 nor PS2160 (which themselves cross). PS1158/PS2160 and PS2052 thus form two biological species that are mirror images of one another at the cellular level. Because type specimens for *A. bodenheimeri* are sinistral, we conclude that this species name applies to PS1158/PS2160. (We will report elsewhere on the identity of the species represented by PS2052.)

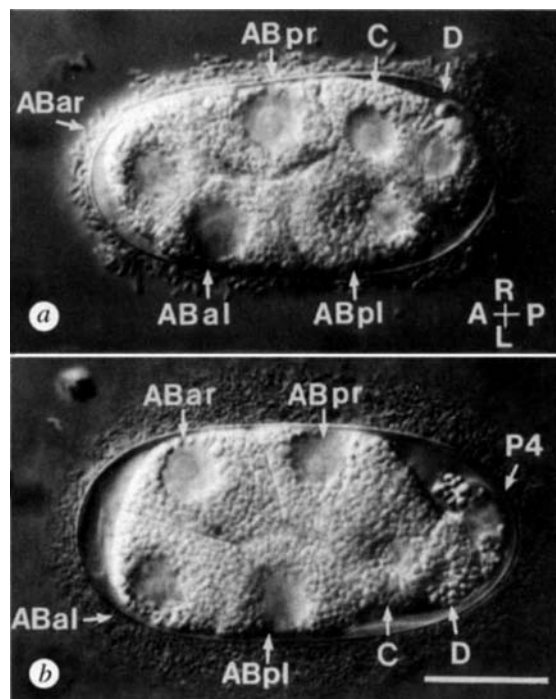
Reverse handedness does not, as in some snails⁵, prevent mating *per se*: mating actually occurred in crosses between PS1158 and PS2052, as revealed by the copulatory plug left by the male over the female's vulva. Also, crosses between sinistral males and rare dextral females from PS1158 yielded progeny, and the converse was also true.

Handedness is apparent from the early cleavages of the nematode embryo, in the

second division of the AB blastomere⁶. The axis of this division can be experimentally inverted in *Caenorhabditis elegans*, causing handedness reversal in the adults⁷. The respective fates of left and right blastomeres are set one cell cycle later by asymmetrical cell interactions⁸. In *Acroboloides* sp. PS2052, as in other dextral nematodes, the left granddaughters of the AB blastomere are positioned anteriorly to their sisters (8/8 embryos). The converse is true in *A. bodenheimeri* PS1158 (8/8) (see figure; in contrast to *C. elegans*, in these species the division of AB is delayed until P1 has divided three times). The asymmetry extends to the next cell cycle in the pattern of contacts to the blastomere MS. This is strikingly similar to the situation in snails, where the cleavage pattern reflects body-plan handedness, and is only rarely reversed^{9,10}.

We believe the present observation to be only the second example of handedness reversal of the whole body plan in an animal phylum. Reversal must be constrained, either because the transition is mechanistically difficult, or because of negative selection on reversed organisms¹¹. Our finding shows that the transition can nevertheless occur and leads to a complete reversal of the population, not only to an equal distribution of handedness.

Developmentally, cleavage handedness could be defined by some chiral cytoskeletal structure^{12–14}. The evolutionary reversal of the cleavage pattern could act at this level, or in the inversion of some component of another embryo axis. We hope that this can be investigated in these



Handedness reversal of the cleavage pattern. Dorsal views of embryos of *Acroboloides* spp. a, PS1158; b, PS2052 at the 9-cell stage. Anterior is to the left of the picture. Cells ABar and ABpr are anterior to their respective sisters ABal and ABpl in PS1158, posterior in PS2052. The other 5 cells at this stage are C, D, P4, MS and E (MS and E are located ventrally). Scale bar, 10 μ m.

closely related nematode species with opposite handedness.

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- zur Strassen, O. *Verh. dt. zool. Ges.* **77**–81 (1951).
- Sulston, J. & Horvitz, H. R. *Dev. Biol.* **56**, 110–156 (1977).
- Steiner, G. *Proc. Helminth. Soc., Wash.* **2**, 74–80 (1936).
- Siddiqi, M. R., De Ley, P. & Khan, H. A. *Afro-Asian J. Nematol.* **2**, 5–16 (1992).
- Lipton, C. S. & Murray, J. *Malacologia* **19**, 129–146 (1979).
- zur Strassen, O. *Zoologica* **107**, 1–142 (1959).
- Wood, W. B. *Nature* **349**, 536–538 (1991).
- Hutter, H. & Schnabel, R. *Development* **120**, 2051–2064 (1994).
- Crampton, H. E. *Ann. N. Y. Acad. Sci.* **8**, 167–169 (1894).
- Vermeij, G. J. *Nature* **254**, 419–420 (1975).
- Gould, S. J. & Young, N. D. *Evolution* **39**, 1364–1379 (1985).
- Guerrier, P. J. *Embryol. exp. Morph.* **23**, 611–637 (1970).
- Meshcheryakov, V. N. & Belousov, L. V. *Wilhelm Roux's Arch.* **177**, 193–203 (1975).
- Freeman, G. in *Time, Space and Pattern in Embryonic Development* 171–196 (Liss, New York, 1983).
- Lorenzen, S. *Veröff. Inst. Meeresforsch. Bremerh. Suppl.* **7** (1981).

SINISTRAL AND DEXTRAL NEMATODES

Designation	Status	Origin	Sinistral*	Dextral*
PS1158	In culture	California	186	14
PS2160	In culture	California	198	2
<i>A. bodenheimeri</i>	Type specimens	Israel	15	1
<i>A. bodenheimeri</i>	Fixed specimens	Libya	10	0
PS2052	In culture	Senegal	2	198
<i>A. bodenheimeri</i>	Fixed specimens	Malawi	0	12

We determined handedness of fixed specimens by the position of the gonad relative to the intestine (these species have a single gonadal arm extending anterior to the vulva). For living populations, handedness was determined on juveniles by the concordance of at least two criteria from ref. 7. Details on the left–right asymmetries at the cellular level and on strain isolations are available on request. Gonad position in the type specimens was not explicitly mentioned by Steiner, but does appear in his original drawings³. Every female of PS1158, irrespective of its phenotype (or of that of the father), produces some dextral progeny, showing that handedness is not imprinted on the embryo by the phenotypic handedness of the mother. The changes in gonad position noted by Lorenzen¹⁵ outside the order Rhabditida could be indicative of changes in handedness of the whole-body plan but may well have evolved independently of handedness.

*Number of animals.

Correction

In the Scientific Correspondence “Turing patterns in fish skin?” by Thomas Höfer and Philip K. Maini (volume 380, page 678), the lettering of two parts of the figure was incorrect: part *b* should have been labelled as part *d*, and part *d* as part *b*. Parts *a* and *c* are labelled correctly.

Hard-headed dualism

Christof Koch

The Conscious Mind: In Search of a Fundamental Theory. By David Chalmers. Oxford University Press: 1996. Pp. 392. \$29.95.

THE most famous twentieth-century philosophical treatise ends with the enigmatic invocation "*Wovon man nicht sprechen kann, darüber muß man schweigen*" ("Whereof one cannot speak, thereof one must be silent"). David Chalmers has obviously considered these words as a challenge and has written a 400-page book (essentially a lengthy version of his PhD thesis) dealing with a topic that the young Ludwig Wittgenstein felt should be left unspoken. As I suspect that Chalmers will be remembered by many as the one who put the so-called 'hard' problem of consciousness on the map (both in the introduction to this book and even more so in his *Scientific American* article in December 1995), let me start here.

Chalmers distinguishes between the 'easy' and the 'hard' problem of consciousness. The 'easy' problem involves giving a reductionist explanation of the many computational or cognitive functions of consciousness (such as sensory discrimination of events in the outside world or in the body itself, memory, self-monitoring global access of information for control, and so on) in terms of their underlying neurobiological implementation. Although the associated research programme might keep scientists busy for a long time, there are no deep epistemological and fundamental mysteries to overcome. Few doubt that such a research programme will eventually succeed in the sense that all functional questions posed in an operational sense (such as what neurons in what part of my brain cause me to make a sensory discrimination or to say "I see red") will have a precise answer. The 'hard' problem involves explaining why seeing a red colour goes hand-in-hand with an ineffable feeling of 'redness' or why pain goes hand-in-hand with a 'painful' feeling. In other words, the 'hard' problem is the core of the mind-body problem: why is it that certain physical systems (such as humans and related species) have subjective states? His distinction between the 'easy' and the 'hard' problem mirrors Ned Block's dichotomy between access consciousness and phenomenal consciousness.

In the natural sciences, this central aspect of our existence has traditionally either been considered to be not amenable to scientific scrutiny or been explained away entirely as a sort of linguistic confusion. The latter position of altogether eliminating consciousness by arguing for its

illusory nature is held most prominently by Daniel Dennett in his *Consciousness Explained* (Little, Brown, 1991). Chalmers rejects this out of hand; his approach is to take the subjective nature of consciousness seriously and to seek a naturalistic explanation. (To anybody who has ever suffered a

tion of every last particle and wave, His work was done. Everything else follows from that (in principle). Everything, that is, except subjective feelings; they do not strictly follow from these facts and laws.

Chalmers marshals a number of different arguments here, evoking those creatures beloved by philosophers, zombies. Chalmers's zombie twin is behaviourally indistinguishable from him; he talks, remembers, moves about and writes books, yet lacks any subjective feelings. The zombie even claims that he is conscious, yet "all is dark inside"; it does not feel like anything to be such a zombie. Chalmers claims that because this golem-like creature can exist in principle, materialism must be incomplete. A second argument holds that it is likewise possible to conceive of a world whose physical facts are identical to ours yet where the qualia are inverted (or different). In such a world, I would have the qualia of 'red' when looking at the sky or the sea (but would, of course, continue to label it as 'blue'). Again, as such a world is logically possible, reductionism fails. Or, as Chalmers states, all the laws of physics, biology and computer science would never lead anybody to predict that Chalmers (or, for that matter, you or I) has a rich, internal life. Experience is an additional ingredient to the physical universe, a tenet Chalmers labels 'naturalistic dualism'.

Mary Evans

Chalmers's dualism is a very mild one and does not invoke any spooky soul-stuff: consciousness arises from the physical but needs an additional set of psychophysical laws as well as so-called 'bridging principles' that allows us to explain how experience maps onto physical states of the conscious system, such as the brain.

The set of these laws, together with a grand unified theory of physics and the initial conditions prevalent at the birth of the Universe, provides a complete description of our Universe.

As a corollary to his view, it seems to follow that pure experience is an epiphenomenon, as zombies can do everything we can do, despite their lack of consciousness. This is not easy for somebody steeped in the tradition of evolutionary biology to accept.

Indeed, it seems to me that the logical possibility of a zombie can be questioned. That Chalmers can imagine a world in which zombies exist does not prove that zombies can exist: it might well be possible that, for reasons unknown to us at the moment, zombies represent a contradiction in terms and can't exist. (In fact, Chalmers is himself careful to stress that zombies almost certainly could not exist in the actual world; rather, his point is that

Shaky concept — the golem in the classic 1920 film of the legend.

root-canal infection on a High Sierra trip, away from painkillers, the illusory nature of the 'bad' experience — or 'qualia' — of this tooth comes as a mighty surprise.)

The central and most demanding part of Chalmers's book involves a closely argued chain of reasoning based on the notion of supervenience. His core argument is that although almost all facts are logical consequences of (or logically supervenient to) physical facts, the exceptions are the phenomenal aspects of consciousness (the 'hard' problem) that are not logical consequences of the physical facts.

The statement that almost all facts are logical consequences of physical facts is an assertion of reductive materialism; as Chalmers expresses it metaphorically, once God fixed the physical laws at the beginning of time, including specifying the posi-

they are conceivable and do not violate any physical principles.) In an analogy suggested by Francis Crick, philosophers might argue that it is possible to conceive of a world without any heat: "what is real is the random movement of molecules against each other". That is, one could argue for the possibility of a world in which statistical physics occurs without the associated thermodynamics. (This argument has nothing to do with our sensation of heat, which is a more complex issue.) Yet temperature is of course an inescapable property of large ensembles of molecules, and playing word games about the conceivability of a world devoid of heat is not helpful for understanding the empirical world around us.

Chalmers attempts to flesh out the bridging principles to include 'structural coherence' and 'organizational invariance'. The first of these principles states that in neuroscientific terms the structure of experience must correspond to the associated psychological processes: the relationships between different hues must correspond to the neuronal substrate of colour representation in the brain. The second principle says that any system with the same fine-grained functional relationship as the various neurons in the brain will show consciousness. It is for these bridging principles that this book should be widely read by anybody trying to fathom the physical basis of consciousness. These are the working hypotheses we need for progress. (For instance, the frequently used commonsense assumption that we can take what people say about their experience as data is such a bridging principle that is, in itself, not directly amenable to tests.)

In the last, speculative part of the book, Chalmers searches for something in the natural universe that can give rise to experience and concludes that 'information' (in the Shannon sense) is responsible. In this view, information has two aspects, physically realizable and phenomenal. And because he seeks simplicity and would not want to introduce an arbitrary threshold for when an information-processing system could be called conscious, he speculates that possibly all such systems have experiential aspects. In this way, my Macintosh computer has experience, as does a ther-

mostat (although he admits that it probably does not feel like a lot "to be a thermostat"). At one fell swoop, almost everything in the Universe would be endowed with qualia — no mean feat. He concludes his book by discussing the possibility of building artificial intelligences that are truly conscious (a proposition that follows directly from his principle of organizational invariance) and by speculating on the relationship between consciousness and various interpretations of the observer in quantum mechanics.

It is at the point where panpsychism and related options are discussed as a viable option that I, as a neuroscientist struggling to come to grips with consciousness, have a further major difference with Chalmers. According to him, experience (or qualia) cannot be dealt with in a reductionist way. A more conservative reaction to Chalmers's framework would be to wait and see and let neuroscience run its course. It might well be possible that, after everything is said and done and we understand

the entire cascade of events leading from light of a certain wavelength impinging on my retina to activation of neurons that correlate with my subjective feeling of 'redness', the 'hard' problem will have disappeared (by analogy to the disappearance of the problem of vitalism). Or we might simply have to accept the fact that the world is as it is and subjective feeling arises out of the physical world in a way that is not further amenable to reductionist, empirical science. To me this seems to be less arbitrary than to populate the entire cosmos with entities who are conscious yet whose consciousness we can never gain access to or know about. Or, to conclude by paraphrasing another giant of the twentieth century, Dirty Harry (as played by Clint Eastwood): "A scientist has gotta know his limitations." □

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Never knowingly undersold

Stephen Battersby

Rain of Iron and Ice: The Very Real Threat of Comet and Asteroid Bombardment. By John S. Lewis. Addison Wesley: 1995. Pp. 236. \$25, £19.95.

The Three Big Bangs: Comet Crashes, Exploding Stars, and the Creation of the Universe. By Philip M. Dauber and Richard A. Muller. Addison Wesley: 1995. Pp. 207. \$25, £20.95.

IN 1911, an Egyptian dog was killed by a meteorite from Mars. This almost farcical piece of bad luck is one of more than a hundred examples of death or damage caused by meteorites that John S. Lewis describes in *Rain of Iron and Ice*.

Lewis's message is that dangerous impacts are much more common than we had thought. About a dozen times a year, US military satellites see an explosion in the upper atmosphere in the 10 to 20 kiloton range, caused by common, fragile asteroids a few metres across. These are not dangerous, but the rarer big or strong bodies that can penetrate the atmosphere are. Lewis makes a case for establishing a system of telescopes to detect near-Earth asteroids and give us enough warning to deflect any body that would be large enough to destroy us.

Other books have warned of the same danger. Recently authors have been encouraged by the collision of the comet Shoemaker-Levy 9 with Jupiter, but stories of death, destruction and great big explosions will probably always be popular. Lewis goes further than usual in dramatizing historical and hypothetical events,

filling one chapter with a series of computer-simulated twentieth centuries, based on his research into the probability of asteroid and comet impact, the behaviour of the different types of body in the atmosphere and the effects of large explosions. Some of these 'scenarios' include impacts larger and vastly more destructive than the best-known impact of the real twentieth century, the Tunguska explosion of 1908, which flattened trees over thousands of square kilometres of Siberia.

He risks indulging the ghoulish in his effort to make the danger graphic, and there are hints of overzealousness elsewhere, even in the title — "Very Real" is surely unnecessary. He is eager to ascribe historical reports to impacts, which may in some cases be a compensation for the reluctance of the establishment, his "Guardians of Truth and Traditional Wisdom", to accept them. He quotes Alexander von Humboldt: "That arrogant attitude of incredulity, which rejects the facts without any attempt to investigate them, is sometimes almost more injurious than unquestioning credulity". What struck (or "impacted on") me were the two words "sometimes almost". I don't think Lewis is unquestioning, but sometimes he seems to ask his audience to be. For example, he reports that in Canterbury in 1178 AD, monks saw the Moon lit up and then darkened by what has been interpreted as an explosion that formed the 20-km crater Giordano Bruno. But by Lewis's calculations (not made explicit) such a big impact on the Moon occurs less than once in a million years. Of course that doesn't mean the

Also bear in mind

Toward a Science of Consciousness edited by S. R. Hameroff, A. W. Kaszlake and A. C. Scott. MIT Press, \$65, £47.50. Over 50 articles arising from the first Tucson conference on consciousness in 1994. Contributors include Chalmers, Koch and Libet.

Conscious Experience edited by T. Metzinger. Imprint Academic, £34, \$51. Collection of 24 essays, with selected bibliography. Contributors include McGinn, Churchland, Chalmers and Dennett.

interpretation is wrong, but it should be enough to justify a degree of scepticism.

Despite the salesmanship and a fondness for lists, his writing is agreeable and his scholarship impressive. There is a lot of good physics and there are hundreds of absorbing historical anecdotes, whose number and similarities tend to be convincing. It is easy to believe that most of them really are accounts of the fireballs and meteorite falls that constitute the small safe end of the impact size distribution. The quantitative estimates of risk in the book are disturbing. It is unfortunate that the uncertainties are not always made clear.

As one of their "Three Big Bangs", Philip M. Dauber and Richard A. Muller provide a shallower but perhaps more disinterested account of the impact hazard, particularly in relation to mass extinctions. Unlike Lewis, they argue for a theory that blames mass extinctions on a hypothetical star called Nemesis, which orbits the Sun every 26 million years or so, causing showers of comets in the inner Solar System each time it nears the Oort cloud.

Supernovae form the second level in the hierarchy of exploding entertainments. The violent ends of massive stars, supernovae are responsible for the creation and distribution of elements heavier than iron in the Galaxy, and so are essential for our existence (a linking theme in the book, but one that could arguably be extended to include just about anything in physics or history). Some carelessness is evident throughout the book — on one page we are told of a star "not too different from our Sun, but perhaps eight to ten times more massive" (I think most astronomers would call that a very different star), and then find out that a neutron star is "held together by the same strong nuclear force that binds nuclei of ordinary matter" (it is held together by gravity).

The biggest bang of all is of course the Big Bang. Here Dauber and Muller have to explain some of the more difficult concepts of cosmology, and although they confuse topology with geometry (a gravitationally closed universe need not be finite, nor an open one infinite) and sometimes seem to be trying to obfuscate rather than explain (an addition of velocities is described as "[scientists] using vector algebra"), here is where they are most successful.

There was promise in the idea, but the book seems a little like three overlong pop-science articles tacked together. A few sensible observations relieve the flow of excited prose, at least. Examining the case for Spaceguard, the system put forward by Lewis and others to detect near-Earth asteroids, the authors do admit that "astronomers would like to see more money spent on telescopes". □

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Is God green?

John Adams

Science, Nonsense and Nonsense: Approaching Environmental Literacy. By Michael Zimmerman. Johns Hopkins University Press: 1995. Pp. 220. \$25.95.

LET us begin with a guessing game. Who said (1) "the account of origins in Genesis is a factual presentation of simple historical truths.... The great Flood described in Genesis... was an historical event, worldwide in its extent and effect" and (2) "God does show anger. When he appeared to Elijah there was earthquake, wind and fire.... God tries to coax and woo, but he also uses disasters"?



Darwin damned — anti-evolution books sold in Tennessee at the start of the Scopes trial in 1925.

Quotation (1) comes from the credo of the Creation Research Society, and is offered by Michael Zimmerman as an example of the sort of nonscientific nonsense proclaimed by creationists in their battle with Darwinian evolutionists. Zimmerman's account of this battle is fascinating and at times frightening. Most readers of *Nature* will not have regular contact with 'creationist scientists', or the other pseudoscientists — homoeopaths, graphologists, astrologers and phrenologists — whose beliefs and activities are examined in this book. It is easy to underestimate their influence. Zimmerman reminds us that the battle did not end with the Scopes 'Monkey Trial' in Tennessee in 1925. As recently as March 1996, the Tennessee legislature narrowly voted down legislation that would have prohibited the teaching of evolution as fact. And Alabama biology texts will soon have to carry a 'health warning' saying that evolution is "a controversial theory".

Zimmerman's two main enthusiasms — defending the environment and denouncing creationists — are brought

together in this book. Creationists, he argues, are harmful to the environment. He produces a few good examples: James Watt, President Ronald Reagan's Interior Secretary, who explained that we do not need to worry about preserving natural resources because the Day of Judgment is fast approaching, after which we won't need them; and others who have denounced environmentalism as the 'new satanism'. But overall the connection is strained. The influence of creationists and other pseudoscientists in debates about environmental issues is exaggerated, and the role of genuine scientific dispute is understated.

Zimmerman preaches environmental salvation through science rather than faith: "once our schools start to turn out scientifically literate individuals, they will be much more likely to be producing environmentalists at the same time, for it is difficult to be the former without being the latter." Would that it were so straightforward.

Most of the environmental *causes célèbres* of the past 25 years — from Alar to global warming — have not featured creationists as the principal participants. They have primarily been debates in which the scientific evidence was inconclusive and both sides could field reputable scientists to argue their cases. Zimmerman's book makes an interesting companion to Aaron Wildavsky's *But is it True? A Citizen's Guide to Environmental Health and Safety Issues* (Harvard University Press, 1995, reviewed in *Nature* 377, 30; 1995). On virtually every issue they disagree. Here is one example from

many. Zimmerman: "[D]ieldrin was banned in the United States because it had been shown to cause, in humans, serious kidney damage, tremors, convulsions, respiratory failure, and central nervous system depression as well as cancerous tumours." Wildavsky: "[T]he concentrations to which the general public is exposed pose no risk to human health.... [The World Health Organization] concludes 'All the available information on dieldrin, including studies on human beings, supports the view that for practical purposes [dieldrin] makes very little contribution, if any, to the incidence of cancer in humans'."

Both are passionate defenders of science. The root of their disagreement lies in their application of the precautionary principle in areas where science has yet to provide clear answers. Zimmerman complains that "instead of forcing companies to demonstrate that the chemicals they manufacture are safe, the government generally assumes that such substances are harmless". Wildavsky makes the opposite complaint;

far too often he insists that "what is not expressly permitted is forbidden; substances or processes must be demonstrated to be benign before they can be used". Zimmerman argues for precaution in the face of possible but unproven danger. Wildavsky argues that "the search for possibilities is endless" and that it diverts scarce resources from more beneficial purposes.

The largest issue addressed by Zimmerman is global warming. He observes that "the scientific community has reached a greater degree of agreement on the issue of global warming than on virtually any other environmental concern.... The scientists [of the Intergovernmental Panel on Climate Change (IPCC)]... conclude that immediate action must be taken." Unfortunately, the degree of agreement is not a satisfactory criterion for resolving scientific disagreements. Had the climatological consensus of the mid-1970s prevailed, and been attached to Zimmerman's version of the precautionary principle, governments around the world would have taken immediate action to warm up the world.

The IPCC example also conspicuously fails to support Zimmerman's attack on the believers in the literal truth of the Old Testament. The author of quotation (2) is Sir John Houghton, chairman of Britain's Royal Commission on Environmental Pollution and co-chairman of the Scientific Assessment Working Group of the IPCC. He was warning that God may persuade us to mend our ways with a disaster. He went on to say ("Me and My God", *Sunday Telegraph*, 10 September 1995) that "[i]f we want a good environmental policy in the future we'll have to have a disaster. It's like safety on public transport. The only way humans will react is if there's an accident." Sir John's view of divine cause and environmental effect would undoubtedly meet Zimmerman's criterion of "superstitious drive!". □

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New Journals

This year, *Nature's* annual New Journals review supplement will appear in the issue of 5 September. Publishers and learned societies are invited to submit journals for review. Journals that first appeared during or after June 1994 and issued at least four separate numbers by the end of May 1996 will be considered. Frequency of publication must be at least three times a year and the main language used must be English. Deadline for submission is 14 June. Please send a least four different issues (the first, the most recent and any two others) of each title to Peter Tallack, *Nature*, Macmillan Magazines Ltd, Porters South, Crinan Street, London N1 9XW, UK. Tel: +44 (0)171 843 4567; e-mail p.tallack@nature.com.

Earth's timekeeper

Joel D. Blum

Radiogenic Isotope Geology. By Alan P. Dickin. *Cambridge University Press*: 1995. Pp. 452. £65, \$89.95.

RADIOACTIVITY is the ultimate geological timepiece. To unravel the complex chronology of the Earth and meteorites (on timescales from years to billions of years), geologists use the products of nuclear transformations that occur in virtually all materials. Some radioactive nuclides with long half-lives have survived from their production in stars before the formation of our Solar System; some are extinct and detectable only by the presence of their daughter nuclides; others are produced in the Earth's atmosphere as it is bombarded by Galactic cosmic rays; and still others are produced as a by-product of nuclear technology.

The field of radiogenic isotope geology borrows from an eclectic mix of disciplines including geology, geochemistry, nuclear chemistry and applied physics. It has grown rapidly over the past 30 years, finding applications throughout the Earth sciences. Until recently, the most comprehensive textbook on the subject was G. Faure's *Principles of Isotope Geology* (Wiley, 1986), which covered both radiogenic and stable isotope geology. Alan Dickin's new book is a worthy successor, filling an important niche by providing an encyclopaedic, research-level review of the principles, methods and applications of radiogenic isotopes in geology.

Dickin clearly and concisely reviews an enormous breadth of literature, and includes 470 figures and nearly 1,200 references. He begins with an introduction to nucleosynthesis and nuclear decay and some common experimental techniques. He then moves on to the main topic of emphasis in the book, the application of radiogenic isotope parent-daughter systems to establishing the age and petrological history of terrestrial igneous, metamorphic and sedimentary rocks. This fits well with the historical approach he takes to explain developments in this field. He also does a commendable job in reviewing methods and applications of studies of rare-gas isotopes, uranium-series isotopes, fission tracks and cosmogenic nuclides, which have found broad applications in fields such as oceanography, atmospheric studies and surface geology. Finally, he discusses the link between isotope geology and astrophysics, reviewing the study of extinct nuclides found in meteorites and their role in establishing the early chronology of the Solar System. The book falls short of being comprehensive only in

its limited focus on some of the newest applications of radiogenic isotopes to hydrogeology, ecology and environmental science.

This is a notable contribution to the scientific literature on radiogenic isotope geology. It provides a readable introduction to applications of radiogenic isotopes for scientists from other disciplines and an important reference for students and researchers working in isotope geology, as well as a glimpse into many of the future directions of the field. □

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Just airborne

C. J. Pennycuik

The Simple Science of Flight: From Insects to Jumbo Jets. By Henk Tennekes. *MIT Press*: 1996. Pp. 137. \$20, £13.95. UK publication, end of June.

HENK Tennekes's theme is that flying animals and aircraft are based on the same physical principles, but, as he explains in the introduction, he regards animal flight as an amusing diversion from the serious work of aeronautical engineering. He extends Crawford H. Greenewalt's classical allometric graphs to show that planes too have weights, wing loadings and speeds that, given the useful properties of logarithmic graph paper, can be strewn about the same line as those of animals. He quotes Vance Tucker's early physiological measurements from the 1960s, and has mined a few more recent sources for weights and wing measurements, which he uses in his graphs and tables without attribution or comment.

The author's Dutch roots and his subsequent career in the United States are betrayed by the brutal mixture of SI, metric non-SI and even ancient British units, which are still used, bizarrely, by American engineers. This does not add to the clarity of his explanations and comparisons. The book is profusely illustrated, mostly by published photographs, which Tennekes or his publisher seems to have scanned into a computer and converted into line drawings. This is visually quite effective, and must save on copyright fees.

Journalists will enjoy rummaging for facts in this book, but those in search of an introduction to either aeronautics or animal flight will find its inadequacies frustrating. □

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Crystal structure of a yeast TFIIA/TBP/DNA complex

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The X-ray crystal structure of the transcription factor IIA (TFIIA) in complex with the TATA-box-binding protein (TBP) and TATA-element DNA is presented at 2.5 Å resolution. TFIIA is composed of a β -barrel and a four-helix bundle motif that together have a boot-like appearance. The β -barrel extends the TBP β -sheet and bridges over the DNA major groove immediately upstream of the TATA box. The four-helix bundle contributes substantially to the surface of the complex available for interaction with additional transcription factors.

TRANSCRIPTION of protein-coding genes in eukaryotes involves the assembly of a preinitiation complex on a TATA-box sequence upstream of the initiation start site¹⁻³. This preinitiation complex contains RNA polymerase II and general transcription factors such as TFIID, TFIIA, TFIIB, TFIIE, TFIIF, TFIIH and TFIIF. TFIID is composed of the TATA-box-binding protein (TBP) and TBP-associated factors (TAFs). TAFs are not required for initiation of basal transcription *in vitro*, but are necessary to respond to transcription activators. The other general factors stabilize the interaction of TFIID with DNA, help recruit RNA polymerase to the preinitiation complex, and facilitate promoter clearance.

TFIIA was initially identified as a general transcription factor required for transcription *in vitro*^{4,5}. It is now generally accepted that TFIIA is not required for accurate initiation of low-level TBP-directed transcription *in vitro*⁶⁻⁹, even though TFIIA stabilizes binding of TBP to the TATA box¹⁰ by direct interaction with TBP and possibly DNA^{11,12}. However, TFIIA will stimulate basal transcription when TFIID is used instead of TBP^{7-9,13-15}. It has been suggested that such stimulation derives from the ability of TFIIA to remove transcription inhibitors such as Dr1 and Mot-1/ADI, which otherwise disrupt the binding of TBP to the promoter^{16,17}. Although such a mechanism may occur, studies using epitope tagging to purify TFIID have established that TFIIA will stimulate TFIID-directed basal transcription in the absence of such inhibitors, implying a more fundamental role for TFIIA in basal transcription⁸. Furthermore, TFIIA is required for high-level stimulation by transcription activators such as VP16, Sp1, NTF and CTF *in vitro*^{14,15,18}. Transcriptional activation by the Epstein-Barr viral activator Zta/Zebra requires TFIIA to mediate Zta-stimulated binding of TFIID to the promoter, presumably through direct contacts of TFIIA with both TFIID and the Zta activation domain¹⁴. TFIIA has also been demonstrated to mediate the action of coactivators such as PC4 and HMG-2 in transcriptional activation^{19,20}. In addition to such *in vitro* results, TFIIA clearly plays an essential role *in vivo*: yeast cells are not viable without it²¹, response to acidic activators in yeast requires interaction between TBP and TFIIA²², and mutations in *Drosophila* TFIIA result in defective photoreceptor development²³.

TFIIA shows striking sequence conservation across yeast, *Drosophila* and humans, despite different subunit compositions. Human and *Drosophila* TFIIA contain three subunits: LN or α , which as a relative molecular mass of $\sim 30,000$ ($M_r \sim 30K$), LC or β ($M_r \sim 20K$), and S or γ ($M_r \sim 13K$); yeast TFIIA contains two subunits: the large subunit, TOA1 ($M_r \sim 32K$), and the small subunit TOA2 ($M_r \sim 14K$)^{7,11}. Cloned human and *Drosophila* LN/ α and LC/ β genes show that both the LN/ α and LC/ β subunits are encoded by

the same gene, the product of which is presumed to be processed post-translationally to yield these two larger subunits²⁴⁻²⁶. Examination of the predicted amino-acid sequences establish that the amino-terminal 54 residues of LN/ α and yeast TOA1 share strong sequence homology, as do the carboxy-terminal 76 residues of LC/ β and yeast TOA1 (ref. 21). The small subunits of all three of these are highly homologous, except for an additional 14 amino acids in the yeast sequence near the C terminus^{8,13-15}. Although all three of the human and *Drosophila* TFIIA subunits and both of the yeast TFIIA subunits are necessary for TFIIA function, only the evolutionarily conserved regions are required for activity, as demonstrated by *in vitro* and *in vivo* studies of yeast TFIIA deletions with the non-conserved yeast TOA1 central sequences removed²⁷.

Previous structural investigations by X-ray crystallography have established that the highly conserved 180-amino-acid C-terminal domain of TBP is a pseudosymmetric saddle-shaped structure, containing a 10-strand β -sheet on its bottom that forms a large hydrophobic concave surface for interacting with DNA, and two α -helices on the top convex surface available for interaction with other factors^{28,29}. When bound to TBP, the TATA-box DNA undergoes significant distortion: the minor groove is flattened and broadened, and the DNA is bent by 80° (refs 30,31). The crystal structure of a heterologous complex containing human TFIIB core, *Arabidopsis* TBP and adenovirus major late promoter DNA shows that the C-terminal core of TFIIB forms two α -helical, cyclin A-type domains, which clamp the C-terminal stirrup of TBP and interact with the phosphate backbone of the DNA both upstream and downstream of the TATA box³².

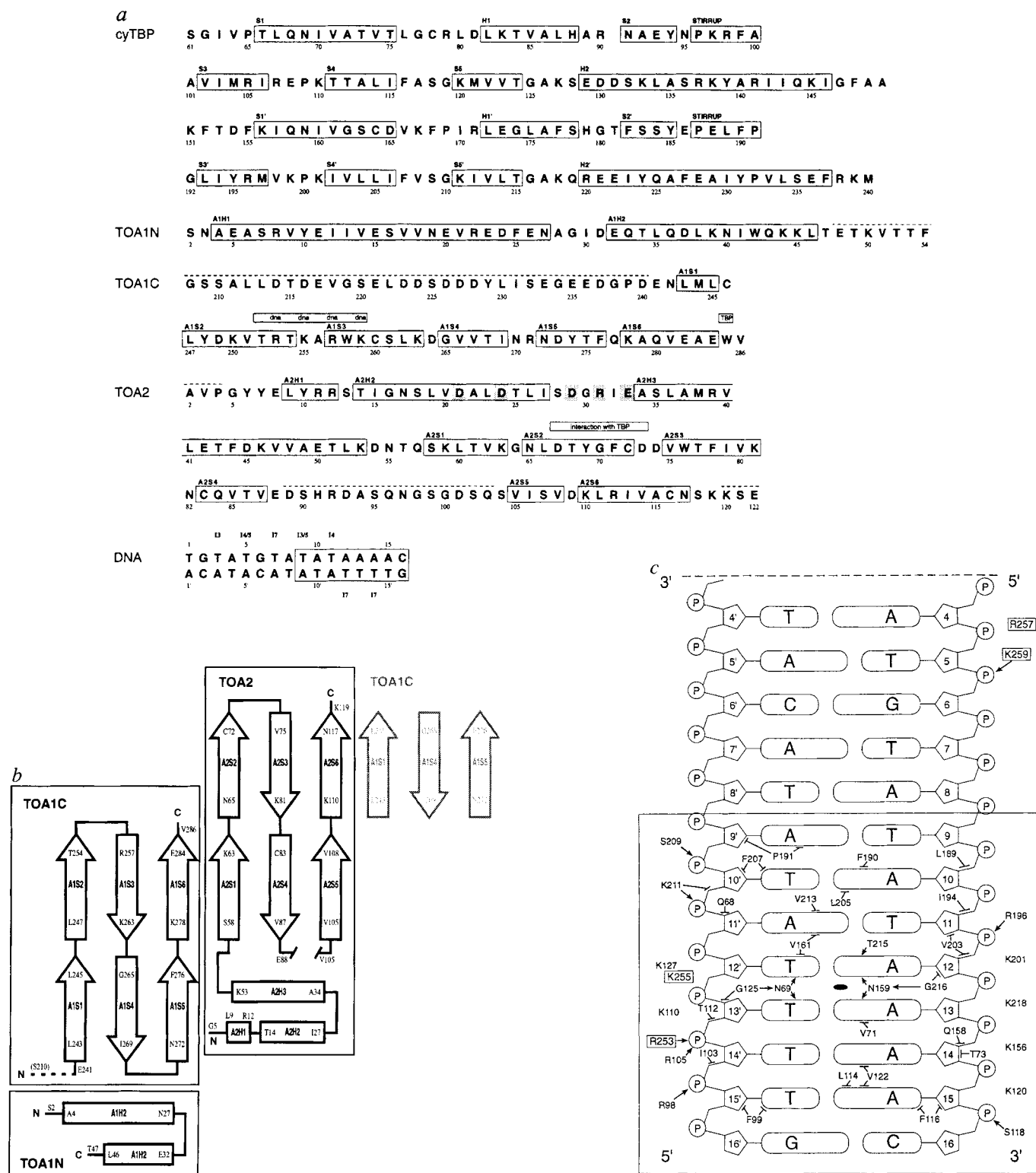
Here we describe the crystal structure of a yeast complex containing the evolutionarily conserved core regions of TFIIA and TBP, and the CYC1 promoter TATA-box DNA at 2.5 Å resolution. This complex reveals the first structure of TFIIA, and shows that the three conserved regions of TFIIA combine to form two domains: a 12-strand β -barrel, and a four-helix bundle. The primary interaction of TFIIA and TBP observed is between two parallel β -strands and results in a 16-strand β -sheet in the complex. TFIIA binds DNA exclusively through phosphate backbone contacts made within the TATA box and with bases upstream of it. Unexpectedly, the orientation of TBP with respect to DNA has changed by slipping two base pairs downstream along the DNA, as compared to the yeast TBP/DNA binary complex.

Structure determination

The chymotrypsin-resistant core containing the three highly conserved regions of yeast TFIIA (yTFIIA) was cloned, overexpressed and purified as three polypeptides corresponding to the N-terminal 54 amino acids of TOA1 (TOA1N), the C-terminal 76

amino acids of TOA1 (TOA1C), and the entire 122-amino-acid TOA2, before reconstitution and purification to yield core yeast TFIIA (cyTFIIA). This cyTFIIA was combined with recombinant core yeast TBP (cyTBP) and a 16-base-pair CYC1 promoter TATA-box-containing DNA fragment, and the ternary complex purified before crystallization (Fig. 1a). The co-crystals grew in space group $P2_12_12_1$ with cell dimensions $59.2 \times 93.1 \times 117.8 \text{ \AA}$, and one ternary complex per asymmetric unit. Although the crystals diffracted to only $3.0 \text{ \AA}$ using a rotating anode X-ray source, diffraction to beyond $2.0 \text{ \AA}$ was observed with synchrotron

radiation. Data were collected to $2.5 \text{ \AA}$ at -170°C on beamline X11 at DESY/EMBL (Hamburg). The structure was solved by multiple isomorphous replacement (MIR), followed by model building incorporating the known cyTBP structure³⁰, and refinement to a final R -factor of 23.5% (free R -factor of 29.7%; Table 1). The electron density was sufficiently clear to build the entire DNA fragment and cyTBP molecule (180 amino acids), and 192 of 251 amino acids of cyTFIIA. Although electron density was apparent for part of the highly negatively charged, N-terminal 31 amino acids of TOA1C, it was not sufficiently well defined to allow



unique assignment. In addition, the non-conserved region covering TOA2 amino acids 89–103 is unobserved.

Overview of the cyTFIIA/cyTBP/DNA complex

The cyTFIIA/cyTBP/DNA ternary complex contains one monomer each of cyTFIIA, cyTBP and DNA (Fig. 2a). The cyTFIIA is shaped like a boot, with overall dimensions $25 \times 35 \times 50 \text{ \AA}$. Its two domains oriented approximately 120° with respect to each other are readily recognized: a 12-strand β -barrel corresponding to the leg of the boot, and a four-helix bundle corresponding to the foot of the boot. The cyTBP is nearly identical to the structure observed in the cyTBP/DNA binary complex with a root-mean-square deviation (r.m.s.d.) for superimposed main chains of $0.46 \text{ \AA}$, excluding the mobile first and last residues. The cyTFIIA molecule binds to the cyTBP/DNA complex such that the top of the TFIIA boot interacts with both the N-terminal stirrup region of cyTBP, and the DNA within and upstream of the TATA box. Viewing a broad-side projection of the complex, as in Fig. 2a, perpendicular to the TBP pseudo-two-fold axis, the TFIIA boot is oriented with its β -barrel cylinder axis nearly parallel to the TBP two-fold, while the pseudo-four-fold axis of the four-helix bundle is approximately perpendicular to it. The conformations of the eight base pairs of TATA-box DNA bound to TBP in both the binary and ternary complexes are essentially identical. The r.m.s.d. of the comparable atoms in their superposition is $0.92 \text{ \AA}$. TFIIA makes minimal contact with DNA by bridging between phosphate chains across the major groove opposite TBP, whereas TBP makes extensive contact, which facilitates its substantial distortion of the TATA-box region.

The cyTFIIA contributes significantly to the exposed protein surface found in the cyTFIIA/cyTBP/DNA complex, as, for its size, it makes minimal contact with cyTBP and DNA. Of the total solvent-accessible surface area ($20,518 \text{ \AA}^2$) observed in the ternary complex, 42% is attributable to TFIIA and 37% to TBP. The

poorly defined, N-terminal region of TOA1C is also likely to cover part of the TBP surface.

Structure of cyTFIIA

The C-terminal 45 amino acids of TOA1C and the C-terminal half of TOA2 each contribute 6 strands to the 12-strand β -barrel forming the upper part of the TFIIA boot, and these pseudo-two-fold symmetrical halves ($0.92 \text{ \AA}$ r.m.s.d. for superposed main-chain atoms) grasp each other in an interlocking fashion (Fig. 2b–d). The β -strands within TOA1C and TOA2 are antiparallel, but the interaction between subunits is made through parallel β -strands (Figs 1b and 2d). The β -barrel is itself held together by a core of conserved hydrophobic residues which fill it completely (Fig. 3a) and result in a broader diameter at the top, with tapering to the bottom. No hydrophilic residues are found in the interior of the barrel, although hydrogen-bonding groups line its ends. The 15 amino acids of hydrophilic sequence between strands 4 and 5 of TOA2 are unobserved in the structure. They correspond closely to the 14 extra and non-essential residues of the yeast small subunit compared to the human and *Drosophila* small subunits²⁷. We suggest that this region forms a flexible and disordered loop between the two β -strands.

The four-helix bundle is built from an antiparallel helix–loop–helix unit comprising TOA1N, which is packed at an angle of 50° against a second antiparallel helix–loop–helix from the N-terminal half of TOA2. Conserved hydrophobic residues form the interface between helices (Fig. 2c), whereas the two loops connecting pairs of helices are located at the toe of the boot, away from the β -barrel.

The β -barrel and four-helix bundle domains are bonded covalently by the TOA2 backbone at residues Asn55 and Thr56. Although we have excluded the 155-amino-acid non-conserved linker sequence encoded in the TOA1 gene from our crystals, several non-covalent interactions link the TOA1N and TOA1C subunits. Hydrogen bonds occur between TOA1N Glu5 and TOA1C Thr275, and TOA1N Tyr10 and TOA1C Glu273 in helix A1H1 and bottom strand A1S5, respectively, in addition to hydrophobic interactions that together fix helix A1H1 to the base of the β -barrel. Furthermore, TOA2 residues 6–13 form an extension (short shoelace) containing a single turn of α -helix (A2H1), which packs against hydrophobic residues of TOA1C on the exterior of the β -barrel to fix the top half of the four-helix bundle against the β -sheet (Figs 2b and 3a). The generally high sequence conservation for TFIIA subunits across yeast, *Drosophila* and human implies that *Drosophila* and human core TFIIA will exhibit the same tertiary structure as for yeast.

The structure of cyTFIIA is fully consistent with biochemical data describing the interactions between the individual subunits for *Drosophila* and human TFIIA. In particular, the N-terminal 59 amino acids of human LN/ α have been shown to be sufficient for binding to the hTFIIA small subunit S/ γ ¹¹. Although it has been suggested^{8,15}, that LC/ β (corresponding to TOA1C) does not interact with the small subunit, it is likely that the use of individually folded subunits makes interpretation of these interaction experiments difficult. TFIIA activity requires the concomitant refolding of both subunits of full-length yTFIIA¹¹, or of all three subunits of cyTFIIA (S.T., unpublished), and it is improbable that the small subunit can fold properly in the absence of at least LC/ β /TOA1C.

Interaction of cyTFIIA and cyTBP

The predominant binding interface observed coupling cyTFIIA to cyTBP is a short stretch of parallel β -sheet formed by the top β -strand A2S2 of TOA2 (amino acids 69 and 71) with the outer β -strand leading to the N-terminal stirrup of cyTBP (amino acids 91, 92 and 94). The resulting continuous, 16-strand β -sheet curves across the underside of TBP bound to DNA and continues as the exposed surface of the TFIIA β -barrel (Fig. 2a). The main-chain interactions of the β -strands are supplemented by hydrogen bonds and van der Waals interactions between TBP, TOA2 and TOA1C

FIG. 1 a, Yeast TBP, TFIIA (TOA1N, TOA1C and TOA2 subunits) and DNA sequences used. Secondary structure assignments determined using the Kabsch/Sander algorithm⁴⁷ are indicated by boxes and labelled H for α -helices, and S for β -strands. Secondary-structure elements of yTFIIA subunits TOA1 and TOA2 contain the prefixes A1 and A2, respectively. Sequences of cyTFIIA that interact with TBP and DNA are marked by boxes around the appropriate sequences. Mutations that affect transcriptional activity without disrupting TBP or DNA interaction²⁷ are shown with a grey background. Residues not included in the final model are indicated with dashed bars. The DNA coding strand (numbered 1–16) is shown above the non-coding strand (numbered 1' to 16'). The 8-bp TATA sequence that interacts with cyTBP in the crystal structure is boxed and the sites of iodine derivatives used for MIR structure determination are indicated. b, Schematic representation of cyTFIIA secondary structure; α -helices and β -strands are represented by rectangles and arrows, respectively, and each structural element is labelled according to the convention indicated above. Three of the TOA1C β -strands are duplicated in grey in the upper right (outside the boxes) to illustrate their interaction with TOA2. c, Schematic representation of contacts made by cyTBP and cyTFIIA with DNA as viewed from the minor groove (base pairs 1–3 omitted). TOA1C residues that interact with DNA are shown boxed; unboxed residues belong to cyTBP. Hydrogen bonds are shown as arrows pointing to the hydrogen-bond acceptor; hydrophobic interactions are indicated by flat-edged pointers. cyTBP contacts DNA exclusively within the 8-bp TATA sequence enclosed in the box, whereas TOA1C contacts DNA both within and upstream of the TATA box. In particular, the TOA1C Arg 253 side chain forms hydrogen bonds to the phosphate of TATA-box base 13' on the non-coding strand. This residue adopts an unusual side-chain conformation as a result of van der Waals contact with TOA1C Trp 158 and TBP Arg 105, which force the side chain to bend towards the DNA. TOA1C Lys 255 is positioned to make salt links between the phosphates of TATA-box bases 11' and 12'. Across the major groove, TOA1C Lys 259 forms hydrogen bonds to the phosphate backbone 3-bp upstream of the 5' end of TATA box on the coding strand. Arg 257 is likely to be positioned near the phosphate backbone one base pair further upstream, between bases 4 and 5, but the electron density for the side chain beyond the β -carbon is very weak, presumably owing to multiple positions adopted by the residue.

(Fig. 3b). Although a change in chain direction precludes continuation of the main-chain β -strand interactions to TBP residues preceding Asn 91, the side chain of Asn 91 mimics the main chain by forming hydrogen bonds to the carbonyl oxygen atom of TOA2 Tyr 69. In a cooperative set of interactions, Tyr 69 reaches over the neighbouring TBP β -strand to form a hydrogen bond with the TBP Arg 105 side chain, held in position by its interactions with TBP Glu 93 and a DNA phosphate group. All these aforementioned hydrogen-bond interactions are made within a hydrophobic pocket created by the top end of the TFIIA β -barrel and the N-terminal stirrup of TBP that shields them from solvent.

Additional interactions between TBP and TOA2 are observed for TBP basic residues, Arg 107 and Lys 83, which form a hydrogen bond and salt link to TOA2 residues Asp 67 and Asp 73, respectively. These basic amino acids near the N-terminal two corners of the TBP β -sheet effectively grasp the top of the TFIIA β -barrel at both sides. Elimination of the salt link between yeast TBP Lys 83 and TOA2 Asp 73 in the mutant *toa2-9* (D73A, D74A) causes temperature-sensitive growth *in vivo* and reduced affinity for TBP *in vitro*, although binding to the TBP/DNA complex is apparently unaffected *in vitro*²⁷. The interaction of TBP Arg 107

with the main chain at TOA2 Asp 67 also creates a pocket within the binding interface, which is filled by the penultimate TOA1C Trp 285. The pocket walls are lined with the aliphatic side chains of TBP Arg 105 and Arg 107, and with TOA2 Tyr 69. Hydrophobic interactions are also observed within the TBP/TOA2 interaction site between TBP Asn 95 and TOA2 Phe 71, TBP Leu 87 and TOA2 Cys 72, and TBP Asn 91 and TOA2 Tyr 69.

All the residues of cyTBP and cyTFIIA that promote the protein–protein and protein–DNA interactions described for the ternary complex are conserved or contain conservative mutations between yeast, *Drosophila* and human. We expect that the ternary cTFIIA/cTBP/DNA complexes of *Drosophila* and human will maintain the salient features of the homologous yeast complex.

The electron density for the N-terminal 31 amino acids of TOA1C (210–240) is not well defined, and indicates that this stretch of highly hydrophilic polypeptide is disordered (long shoelace). We expect that the chain before TOA1C Glu 241 loops back along the exposed surface of the TFIIA β -barrel and contacts residues of TBP helix 2 before wrapping around TBP and making contact with the side of TBP helix 1. Our reasoning is as

TABLE 1 Crystallographic analysis

Diffraction data	Native	DACIOD4	DACIOD5	DACIOD3	DACIOD7
X-ray source	synchrotron	synchrotron	synchrotron	rotating anode	rotating anode
Resolution (Å)	20–2.5	20–2.5	20–2.5	20–3.05	20–3.1
Reflections (observations/unique)	196,513/22,476	198,256/23,112	197,840/22,865	123,730/12,737	116,729/12,294
Completeness (%)	97.2	98.6	97.3	99.6	99.6
R_{merge}^*	5.0	3.5	6.0	14.8	5.0
MIR analysis (20–2.5 Å)					
Phasing power		0.95	1.08	0.99	0.90
Cullis R -factor		0.81	0.79	0.84	0.83
Refinement statistics					
Resolution (Å)	6–2.5	mean B -factor (Å ²)	21.5		
R -factor/free R -factor (%)	23.5/29.7	r.m.s.d. bond length (Å)	0.008		
Reflections, $I > 2\sigma$ (working/test)	18,697/2,061	r.m.s.d. bond angle	1.3		
Number of atoms	3,605				

The genes for TOA1 and TOA2 were obtained by PCR amplification of yeast genomic DNA, and the gene products were overexpressed, purified and reconstituted essentially as described²¹. PAGE analysis of a crystal grown from solutions containing full-length yTFIIA/cyTBP/DNA indicated that TFIIA was proteolyzed during crystallization. N-terminal sequencing and mass spectrometry of TFIIA digested with chymotrypsin to mimic the fragments from the crystal showed that the non-conserved amino acids 55–209 were missing. TOA1N[TOA1(2–54)] was overexpressed using the T7 expression system³⁹ and purified by Q-Sepharose (Pharmacia), Phenyl-5PW and DEAE-5PW (TSK). The TOA1 C terminus [TOA1(210–286)] was overexpressed as a glutathione S-transferase (GST) fusion protein, purified under denaturing conditions on Sepharose S300 (Pharmacia) and DEAE-5PW and refolded by dialysis. The N-terminal GST fusion protein was removed by digestion with recombinant tobacco etch virus N1a protease⁴⁰, and the resulting TOA1C [TOA1(210–286)] with an additional Gly–Ser at the N terminus purified on glutathione–Sepharose and DEAE-5PW. The TOA2 polypeptide was overexpressed using the T7 expression system and purified from inclusion bodies on Sepharose S300 and DEAE-5PW under denaturing conditions. TOA1N, TOA1C and TOA2 were combined in equimolar ratios in 7 M urea and refolded by dialysis before purification on DEAE-5PW (TSK). The resulting cyTFIIA behaves as a heterotrimer containing one copy each of TOA1N, TOA1C and TOA2, as judged by size-exclusion chromatography on Superdex 200 HR (Pharmacia) and dynamic light scattering (Protein Solutions), consistent with the observation that yTFIIA lacking non-conserved TOA1 sequences is a functional heterodimer²⁷. Core yeast TBP (residues 61–240) was overexpressed using the T7 expression system and purified on heparin–Sepharose and CM-3SW (TSK). Oligonucleotides corresponding to CYC1 promoter –129 to –114 (numbered from the translation start site) were synthesized incorporating 5-iodo-dU phosphoramidite (Glen Research) where indicated, and purified on Nucleosil 300-5 C8 (Macherey–Nagel). cyTFIIA, cyTBP and DNA were combined in equimolar ratios and purified by size-exclusion chromatography on Superdex 200 HR before concentrating against (in mM) 2 bis-Tris, pH 7.0, 10 KCl, 0.1 EDTA, 0.5 DTT. The concentrated ternary complex was monodisperse in solution with an estimated M_r of 55K (expected M_r of 59K for one copy each of cyTBP, cyTFIIA and DNA), as determined by dynamic light scattering. Crystals were grown using 8- μ l hanging drops containing 5 mg ml^{–1} or 85 μ M complex against (in mM) 25 sodium acetate, pH 5.5, 100 NH₄Cl, 10 CaCl₂, 1 DTT, 8–10% PEG6000 at room temperature. Crystals were flash-cooled by transferring through the same buffer (with 12–13% PEG6000) augmented stepwise to 5, 10, 15, 20 and 25% glycerol, mounting in a tapered glass capillary, and flash immersing in liquid nitrogen. Data were collected on a MAR image plate using a Rigaku FR-C rotating anode X-ray generator or synchrotron radiation at the X11 beamline at EMBL Hamburg Outstation, c/o DESY, and processed using local programs (T.J.R., unpublished). MIR phases calculated with MLPHARE⁴¹ using 3 doubly and 1 triply iodinated derivatives from 20 to 2.5 Å produced an overall figure of merit of 0.46. Cross-difference Fourier maps identified the iodine sites of each derivative unambiguously enabling the positioning of a DNA model and cyTBP (allowing for the 2-bp shift) based on the 1.9-Å yeast TBP/DNA structure (PDB accession number 1YTB). Iterative rounds of model building with O⁴² and phase combination with SIGMAA⁴³ created a model refined by simulated annealing (XPLOR⁴⁴) and conjugate gradient methods (TN⁴⁵). The final model includes the entire cyTBP molecule, TOA1N(2–47), TOA1C(241–286), TOA2(5–88, 104–119) and the 16-bp DNA. There are 4 of 372 outliers in a Ramachandran plot, including Lys 255 which interacts with DNA and for which there is clear electron density.

* $\sum_{hkl} \sum_i |I_{hkl,i} - \bar{I}_{hkl}| / \sum_{hkl} \sum_i I_{hkl,i}$, where $\bar{I}_{hkl}$ is the mean of measurements for a single hkl .

follows: (1) weak, but mainly continuous electron density exists along this path; (2) ample space exists in the crystal to accommodate this course of the polypeptide chain; (3) basic residues line the proposed path for the missing TOA1C chain, and would complement its frequently occurring acidic side chains (Fig. 3c); (4) TOA1C residues 217–240 were found to be necessary for the formation of stable TFIIA/TBP and TFIIA/TBP/DNA complexes²⁷; and (5) basic amino acids in TBP helix 2, in particular Lys 138, have been identified by genetic and biochemical screens to be important for interaction with TFIIA^{22,33–35}. The abundance of glycine, serine, aspartate and glutamate in the N terminus of TOA1C may dictate a flexible and highly hydrated peptide chain displaying several conformations and thereby attenuating the electron density. Attempts to anneal this stretch of chain by slow cooling the crystal to provide a more identifiable conformation are in progress.

Because TFIIA interacts with the N-terminal domain of the pseudosymmetric TBP molecule partly through main-chain β -strand interactions, we have considered how discrimination from C-terminal domain binding is achieved. In either case, the three inter-main-chain hydrogen bonds would be conserved, as would some side-chain interactions, such as TOA2 Tyr 69 with TBP Arg 196 instead of Arg 105. However, the embracing interactions of TBP Arg 107 and Lys 83 would be eliminated, as the equivalent residues on the C-terminal half of TBP are Val 198 and Glu 173. Potentially more deleterious is the steric hindrance that would result because of the one additional amino acid in the

TBP C-terminal half. TBP Thr 181 is located between helix 1 and strand 2, the outer strand leading to the stirrup, and in our attempts to dock TFIIA with TBP at this site, Thr 181 makes unacceptably close contact to TFIIA. A substantial conformational rearrangement would be required to relieve interference due to the helix and strand of TBP, possibly disrupting close packing within TBP. Discrimination between the two halves of TBP by TFIIA may be one reason for the highly conserved extra residue in the C-terminal half of TBP.

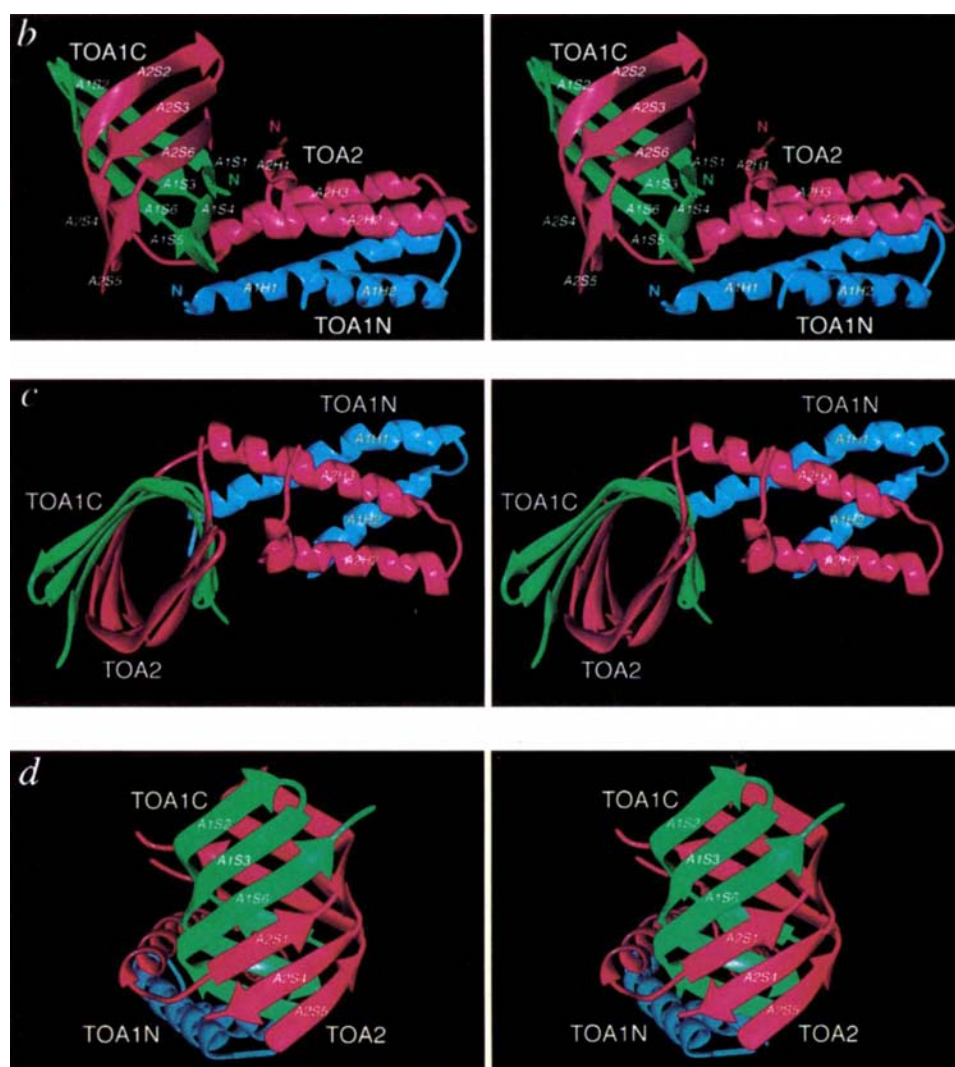
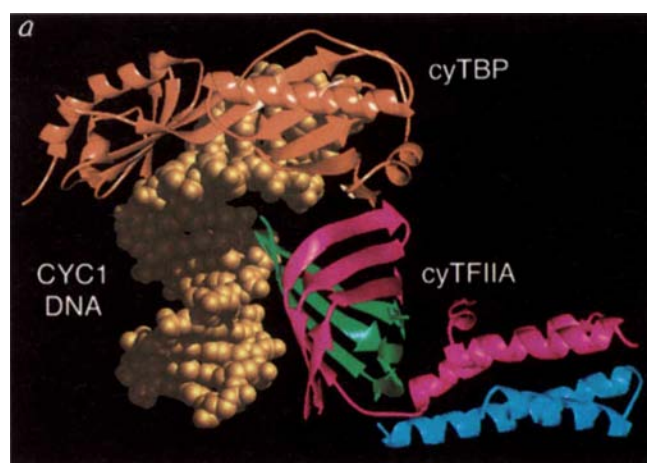


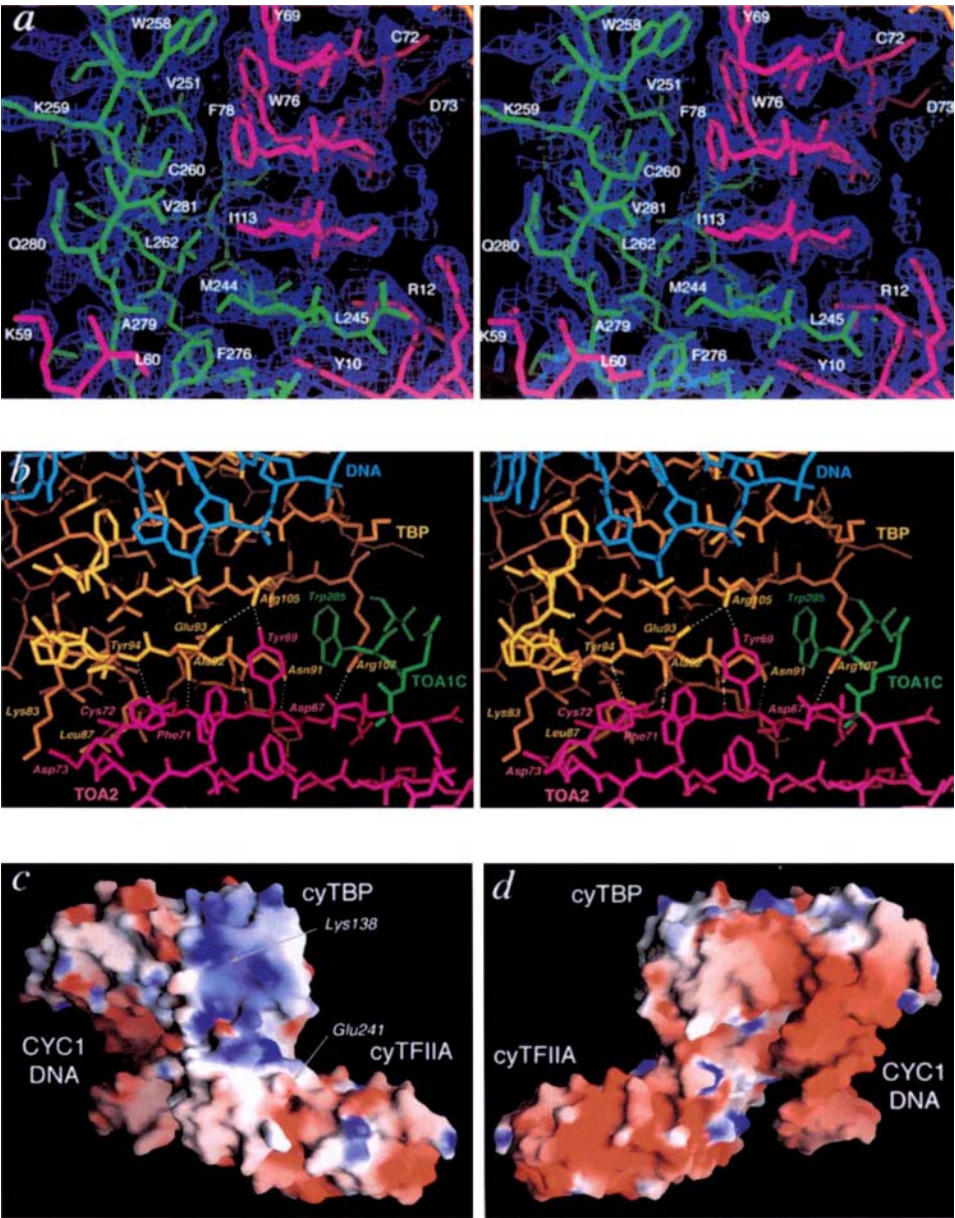
FIG. 2 Structure of cyTFIIA/cyTBP/DNA complex and details of cyTFIIA structure. *a*, Ribbon and space-filling representation of the ternary complex viewed from one side of the TFIIA boot. cyTFIIA is composed of the N terminus of the large subunit TOA1 (blue), the corresponding C terminus (green), and the small subunit TOA2 (red); cyTBP is shown in orange and DNA in yellow. The same colour scheme is used for Figs 3 and 4. cyTFIIA interacts with cyTBP through a parallel β -strand interaction, and with DNA through the loop region between strands A1S2 and A1S3 of TOA1C. *b*, Stereo ribbon representation of cyTFIIA with secondary-structure elements labelled appropriately, viewed from the side of the TFIIA boot (same view as *a*). *c*, Stereo ribbon representation of cyTFIIA viewed from the top of the TFIIA boot, showing the interior of the β -barrel, as well as the interaction between TOA1N and TOA2 in the four-helix bundle. *d*, Stereo ribbon representation of cyTFIIA viewed from the back of the TFIIA boot showing the parallel β -strand interaction between TOA1C and TOA2 on one side of the β -barrel. Figures were created using MIDAS⁴⁸.

TABLE 2 DNA helix parameters

Helical twist (deg)	28.4	34.2	39.0	24.3	35.2	37.1	27.0	31.2	20.3	20.3	23.6	9.9	21.0	21.4	33.1		
Roll (deg)	-1.1	0.6	7.0	2.8	7.3	0.7	7.1	0.3	50.6	18.2	10.1	20.6	22.5	15.1	37.5		
Slide (Å)	0.66	-0.70	-1.80	-1.04	-1.52	-1.28	-1.32	-0.94	-1.18	-0.82	1.77	1.00	1.14	0.15	-1.89		
Rise (Å)	3.12	3.09	3.46	3.19	3.72	3.42	3.11	3.35	5.98	3.84	3.05	3.62	3.97	3.34	5.84		
Tilt (deg)	-11.18	1.84	-1.22	2.65	-0.46	0.94	0.26	3.86	-6.42	2.95	2.06	-4.22	3.34	-0.86	-3.08		
P-P distance (Å)		6.98	7.15	6.39	7.21	6.79	5.93	5.83	5.76	6.14	5.55	5.88	5.61	6.46	6.09		
Base pair	5' 3'	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
		T	G	T	A	T	G	T	A	T	A	T	A	A	A	A	C
		A	C	A	T	A	C	A	T	A	T	A	T	T	T	T	G
		-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	-11	-12	-13	-14	-15	-16
P-P distance (Å)		6.26	7.11	7.04	5.97	6.75	6.75	7.05	6.58	6.07	6.07	6.03	6.03	6.09	6.01		
Minor groove width (Å)		7.39	5.36	6.07	5.90	4.09	5.33	6.11	7.35	8.09	9.09	8.95	9.24	8.09	7.04		
Buckle (deg)		-41.0	-17.6	8.5	-1.2	21.6	-39.8	-49.4	-17.6	0.0	-41.4	-27.9	3.2	38.6	59.6	72.0	3.7
Propeller twist (deg)		-2.7	-12.0	-7.4	-8.7	-6.6	-12.2	-11.3	-12.0	-0.5	18.5	-11.3	-1.8	0.9	-5.8	-8.3	2.1

DNA helix parameters for the CYC1 DNA in the ternary complex were calculated using CURVES⁴⁶. The minor groove width is defined as the shortest O4'-O4' distance minus 2.8 Å to account for the van der Waals radius of the oxygen atoms^{30,31}.

FIG. 3 Details of interactions in the cyTFIIA/cyTBP/DNA complex. *a*, Stereo 2F_o - F_c electron density (contour 1.3σ) for the interior of the cyTFIIA β-barrel, showing the hydrophobic packing between TOA1C (green) and TOA2 (red). The side chains of TOA1C and TOA2 form complementary hydrophobic surfaces within the β-barrel which preclude the formation of TOA1C or TOA2 homodimers. The hydrophobic interaction between TOA1C Leu 245 on the outside of the β-barrel and Tyr 10 and Arg 12 in the short TOA2 α-helix A2H2 are seen in the lower right. *b*, Stereo view of the interaction between cyTBP, TOA2 and TOA1C. The colours are as Fig. 2, except that DNA has been shown in blue for clarity. Hydrogen bonds are shown by broken lines. Main-chain β-strand hydrogen bonds are observed between TBP Tyr 94 N and TOA2 Phe 71 O, TBP Ala 92 O and TOA2 Phe 71 N, and TBP Asn 91 N and TOA2 Tyr 690. Description of the other electrostatic and hydrophobic interactions are provided in the main text. The accessible surface area buried in the interaction between cyTBP/DNA, cyTFIIA/cyTBP and cyTFIIA/DNA are 3,060, 1,260 and 480 Å², respectively. TFIIA binds the TBP/DNA complex with an apparent binding constant of 20 pM (100 times stronger than TBP binds DNA¹¹). *c*, GRASP⁴⁹ representation of cyTFIIA/cyTBP/DNA molecular surface colour coded for electrostatic potential in the range of -10k_BT (red) and +10k_BT (blue), where k_B is the Boltzman constant, and T is the absolute temperature. Similar view to Fig. 2a is shown here, the toe of TFIIA boot rotated towards the viewer. The location of TOA1C Glu 241 just after the uninterpreted electron density (not shown) for the N-terminal 31 residues for TOA1C is indicated. Weak density is present in the positively charged (blue) channel created by the TBP/TFIIA interface and by basic residues in helix H1 of TBP critical for binding to TFIIA. *d*, GRASP representation of the ternary complex as in *c*, rotated approximately 120° about the vertical axis. The four-helix bundle creates a large negatively charged solvent-accessible surface along the side of the TFIIA boot.



DNA interactions

The interactions of cyTBP with DNA in the cyTFIIA/cyTBP/DNA complex are very similar to those in the 1.8 Å resolution cyTBP/DNA crystal structure, with one essential difference: TBP in the ternary complex has shifted two base pairs downstream compared to the binary complex. In the ternary complex, TBP is positioned over the sequence TATAAAAC contained in a 16-base-pair fragment, compared with TATATAAA in the 29-base hairpin used for the binary complex³⁰. Despite this shift, TBP forms almost identical hydrogen bonds and hydrophobic contacts with the 8-base-pair binding site (Fig. 1c). The exceptions occur for the contacts of TBP Asn 69, Asn 159 and Thr 124 with TATA-box position 5, which is a T·A base pair in the binary complex and an A·T base pair in the ternary complex. The Asn 69 and Asn 159 residues make an equivalent number of hydrogen bonds with the DNA, whereas Thr 124 makes no apparent contact with DNA in the ternary complex.

The shift in position observed in crystal structures for TBP bound to the CYC1 TATA box may be induced by TFIIA binding, or represent an equilibrium between the two sites in solution and subsequent selection by crystallization. The DNA fragment is sufficiently long in both cases to avoid bias. Although we cannot rule out either alternative, the TBP binding site used in the ternary complex is similar to the TATAAAAG binding site in the adenovirus major late promoter used for the *Arabidopsis* binary complex crystal structure³¹, and as a consequence of the run of TATATA in the CYC1 promoter, two overlapping TATA-box sequences consistent with the consensus TATAa/tAa/t³⁶ occur.

DNase I footprinting suggested that TFIIA contacts DNA over 4 or 5 bases upstream of the TATA box, although the possibility that TFIIA induces a conformational change in TBP that extends its DNA interface could not be excluded³⁷. The cyTFIIA/cyTBP/DNA structure shows that cyTFIIA contacts DNA directly without significantly altering the cyTBP/DNA structure. Whereas TOA2 is responsible for most of the TFIIA/TBP β -sheet interactions, only the TOA1C basic residues Arg 253, Lys 255, Arg 257 and Lys 259 make direct contact with DNA. These four residues were previously identified from alanine scanning mutants²⁷. They are located on the top, back of the TFIIA boot, near and within the loop connecting β -strands A1S3 and A1S4, and span the major groove, contacting the phosphate backbone within the TATA box at positions 11'–13' on the non-coding strand and again 3–5 base pairs upstream from the TATA box on the coding strand. These combined interactions should be sensitive to major groove width, and therefore the DNA sequence immediately upstream of the TATA box may affect complex formation. The occurrence of hydrogen bonds mediated through a single water molecule to the DNA major-groove surface appears unlikely, although assignment of water molecules at higher resolution may show that further significant interactions to phosphate groups occur (for example, from Thr 252 and Thr 254). TFIIA probably binds DNA only in the presence of TBP because of a requirement for the TBP-induced DNA distortion.

Implications for transcription

We do not observe any significant conformational difference in the TBP/DNA structure within the cyTFIIA/cyTBP/DNA complex (Table 2) as compared to the cyTBP/DNA complex, aside from the shift by two base pairs of cyTBP along the DNA. TFIIA binding to TBP/DNA has been proposed to cause a conformational change in full-length TBP that involves its N-terminal extension³⁴, but this is not addressed by the ternary complex structure, as cyTBP lacks this sequence. It is unlikely that the shift of two base pairs plays a role in this binding study because the adenovirus major late promoter used is not degenerate like the CYC1 promoter, but flanked by G·C base pairs on either side.

Because core TFIIIB binds to the C-terminal stirrup of core TBP directly opposite the TBP molecule from the TFIIA binding site, no obvious steric hindrance would occur to prevent formation of

the quaternary complex cTFIIIB/cTFIIA/cTBP/DNA (Fig. 4b), and such a complex containing intact molecules has been detected *in vitro*³⁸. However, it is not clear from the crystal structures of chTFIIIB/*Arabidopsis* TBP/DNA and cyTFIIA/cyTBP/DNA complexes whether TFIIIB and TFIIA would interact in the quaternary complex without additional conformational changes.

The cyTFIIA/cyTBP/DNA complex makes available a large area of protein surface accessible to interactions with other transcription factors. For example, the TBP/TFIIA junction creates a continuous surface that would allow cooperative binding of more transcription factors in the preinitiation complex. Another potential binding site is the negatively charged, exposed surface of the TFIIA four-helix bundle (Fig. 3d). Mutations in the TOA2 subunit, which map to the toe of the TFIIA boot, cause temperature-sensitive growth in yeast and severely diminish

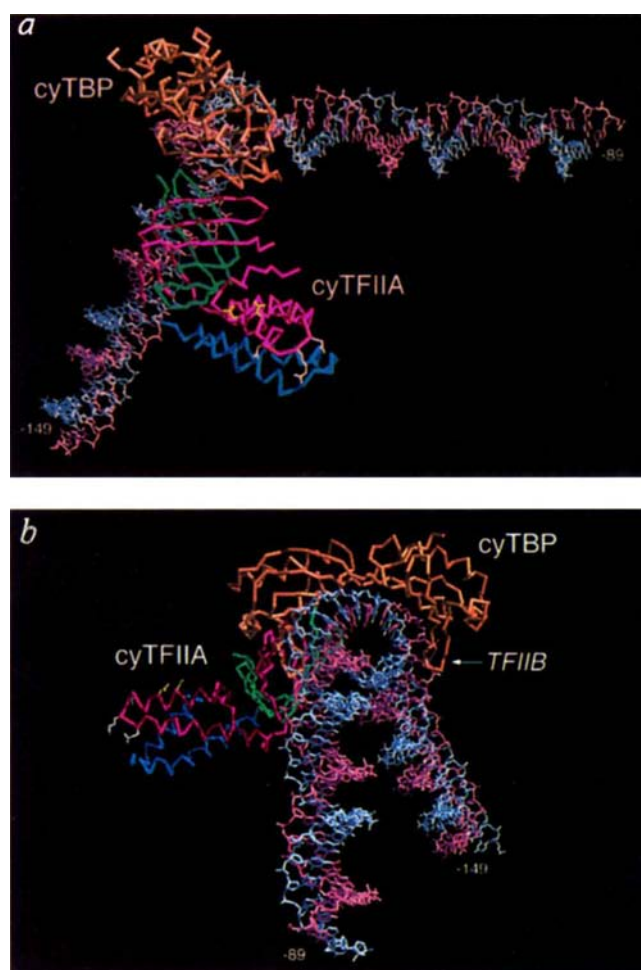


FIG. 4 cyTBP and cyTFIIA interacting on extended CYC1 DNA. **a**, View of cyTFIIA and cyTBP on DNA extended on both ends as hypothetical B-form DNA. The DNA is numbered from the start of translation because of the multiple start sites between positions -93 and -28 on the CYC1 promoter⁵⁰. The coding strand is shown in light blue, and the non-coding strand in pink. The side chains of potentially critical residues of cyTFIIA identified by alanine-scanning mutational analysis²⁷ are shown on the α -carbon tracing. The two yellow aspartic acid residues correspond to the *toa2-3* mutation (TOA2 D21A/D24A), while the three white residues correspond to the *toa2-5* mutation (TOA2 D29A/R31A/E33A). These mutations do not affect the ability of TFIIA to interact with TBP or with DNA, but cause temperature-sensitive growth *in vivo* and reduced transcriptional activity *in vitro*. **b**, Alternative view of the ternary complex on extended DNA perpendicular to the cyTBP approximate two-fold symmetry axis. The location of TFIIIB binding to the C-terminal stirrup of TBP is indicated³².

transcriptional activity *in vitro*²⁷. One of the two mutants characterized, *toa2-5* (D29A/R31A/E33A), maps to the loop joining the two antiparallel TOA2 α -helices, and the other more deleterious *toa2-3* (D21A/D24A) occurs along the top side of the

TOA2 α -helix A2H2 (Fig. 4a). Because these mutations do not affect the ability of TFIIA to bind TBP/DNA, it is likely that the four-helix bundle binds additional factors, such as TAFs or coactivators necessary for activated transcription. □

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1. Maldonado, E. & Reinberg, D. *Curr. Opin. Cell Biol.* **7**, 352–361 (1995).
2. Hori, R. & Carey, M. *Curr. Opin. Genet. Dev.* **4**, 236–244 (1994).
3. Conaway, R. C. & Conaway, J. W. *A. Rev. Biochem.* **62**, 161–190 (1993).
4. Matsui, T., Segall, J., Weil, P. A. & Roeder, R. G. *J. biol. Chem.* **255**, 11992–11996 (1980).
5. Reinberg, D., Horikoshi, M. & Roeder, R. G. *J. biol. Chem.* **262**, 3322–3330 (1987).
6. Sayre, M. H., Tschochner, H. & Kornberg, R. D. *J. biol. Chem.* **267**, 23376–23382 (1992).
7. Cortes, P., Flores, O. & Reinberg, D. *Molec. cell. Biol.* **12**, 413–421 (1992).
8. Sun, X., Ma, D., Sheldon, M., Yeung, K. & Reinberg, D. *Genes Dev.* **8**, 2336–2348 (1994).
9. Hansen, S. K. & Tjian, R. *Cell* **82**, 565–575 (1995).
10. Imbalzano, A. N., Zaret, K. S. & Kingston, R. E. *J. biol. Chem.* **269**, 8280–8286 (1994).
11. Ranish, J. A. & Hahn, S. *J. biol. Chem.* **266**, 19320–19327 (1991).
12. Coulombe, B., Li, J. & Greenblatt, J. *J. biol. Chem.* **269**, 19962–19967 (1994).
13. De Jong, J., Bernstein, R. & Roeder, R. G. *Proc. natn. Acad. Sci. U.S.A.* **92**, 3313–3317 (1995).
14. Ozer, J. *et al. Genes Dev.* **8**, 2324–2335 (1994).
15. Yokomori, K. *et al. Genes Dev.* **8**, 2313–2323 (1994).
16. Inostroza, J. A., Mermelstein, F. H., Ha, I., Lane, W. S. & Reinberg, D. *Cell* **70**, 477–489 (1992).
17. Auble, D. T. & Hahn, S. *Genes Dev.* **7**, 844–856 (1993).
18. Wang, W., Gralla, J. D. & Carey, M. *Genes Dev.* **6**, 1716–1727 (1992).
19. Ge, H. & Roeder, R. G. *Cell* **78**, 513–523 (1994).
20. Shykind, B. M., Kim, J. & Sharp, P. A. *Genes Dev.* **9**, 1354–1365 (1995).
21. Ranish, J. A., Lane, W. S. & Hahn, S. *Science* **255**, 1127–1129 (1992).
22. Stargell, L. A. & Struhl, K. *Science* **269**, 75–78 (1995).
23. Zeidler, M. P., Yokomori, K., Tjian, R. & Mlodzik, M. *Genes Dev.* **10**, 50–59 (1996).
24. De Jong, J. & Roeder, R. G. *Genes Dev.* **7**, 2220–2234 (1993).
25. Ma, D. *et al. Genes Dev.* **7**, 2246–2257 (1993).
26. Yokomori, K., Admon, A., Goodrich, J. A., Chen, J. L. & Tjian, R. *Genes Dev.* **7**, 2235–2245 (1993).
27. Kang, J. J., Auble, D. T., Ranish, J. A. & Hahn, S. *Molec. cell. Biol.* **15**, 1234–1243 (1995).
28. Nikolov, D. B. *et al. Nature* **360**, 40–46 (1992).
29. Chasman, D. I., Flaherty, K. M., Sharp, P. A. & Kornberg, R. D. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8174–8178 (1993).

30. Kim, Y., Geiger, J. H., Hahn, S. & Sigler, P. B. *Nature* **365**, 512–520 (1993).
31. Kim, J. L., Nikolov, D. B. & Burley, S. K. *Nature* **365**, 520–527 (1993).
32. Nikolov, D. B. *et al. Nature* **377**, 119–128 (1995).
33. Buratowski, S. & Zhou, H. *Science* **255**, 1130–1132 (1992).
34. Lee, D. K., DeJong, J., Hashimoto, S., Horikoshi, M. & Roeder, R. G. *Molec. cell. Biol.* **12**, 5189–5196 (1992).
35. Tang, H., Sun, X., Reinberg, D. & Ebright, R. H. *Proc. natn. Acad. Sci. U.S.A.* **93**, 1119–1124 (1996).
36. Breathnach, R. & Chambon, P. *A. Rev. Biochem.* **50**, 349–383 (1981).
37. Hahn, S., Buratowski, S., Sharp, P. A. & Guarente, L. *EMBO J.* **8**, 3379–3382 (1989).
38. Buratowski, S., Hahn, S., Guarente, L. & Sharp, P. A. *Cell* **56**, 549–561 (1989).
39. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. *Meth. Enzym.* **185**, 60–89 (1990).
40. Parks, T. D., Leuther, K. K., Howard, E. D., Johnston, S. A. & Dougherty, W. G. *Analyt. Biochem.* **216**, 413–417 (1994).
41. Otwinowski, Z. in *Isomorphous Replacement and Anomalous Scattering* (eds Wolf, W., Evans, P. R. & Leslie, A. G. W.) 80–86 (SERC, Daresbury Laboratory, Warrington, UK, 1991).
42. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. *Acta crystallogr.* **A47**, 110–119 (1991).
43. Read, R. J. *Acta crystallogr.* **A42**, 140–149 (1986).
44. Brünger, A. X-PLOR Version 3.1 (Yale University, New Haven, CT, 1992).
45. Tronrud, D. E. *Acta crystallogr.* **A48**, 912–916 (1992).
46. Lavery, R. & Sklenar, H. *J. biomol. Struct. Dynam.* **6**, 63–91 (1988).
47. Kabsch, W. & Sander, C. *Biopolymers* **22**, 2577–2637 (1983).
48. Ferrin, T. E., Huang, C. C., Jarvis, L. E. & Langridge, R. J. *molec. Graph.* **6**, 13–27 (1988).
49. Nicholls, A., Sharp, K. & Honig, B. *Proteins* **11**, 281–296 (1991).
50. Li, W. & Sherman, F. *Molec. cell. Biol.* **11**, 666–676 (1991).

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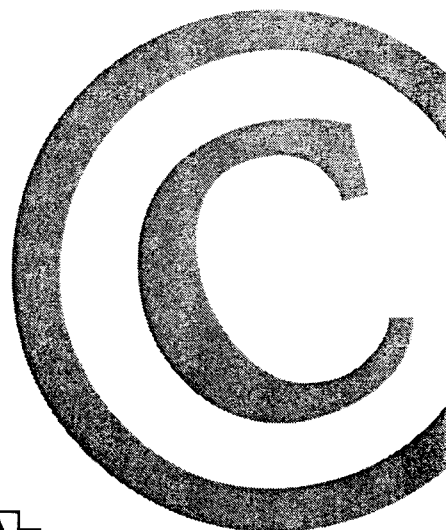
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Single optical photon detection with a superconducting tunnel junction

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THE charge-coupled device (CCD) has become the detector of choice in optical astronomy. CCDs provide a very linear response to detected photons, are very efficient at some wavelengths, and can now provide coverage of a relatively wide field of view¹⁻³. But they become quite inefficient with decreasing wavelength, and they lack intrinsic wavelength and time resolution. The only way to select specific wavelengths is to place filters in front of the detector, which makes the total system less efficient. Time resolution can be achieved only with short exposures, which are possible only with very bright sources. Here we report a superconducting device that can overcome these limitations, and which has performance characteristics far superior to existing photon counting systems⁴⁻⁷. Our superconducting tunnel junction can detect individual photons at rates up to 2.5 kHz in the wavelength range 200–500 nm, with an intrinsic spectral resolution of 45 nm and a quantum efficiency estimated to be about 50 per cent. The theoretical resolution of the present device is ~ 20 nm, but use of superconductors with lower transition temperature could improve that to 8 nm.

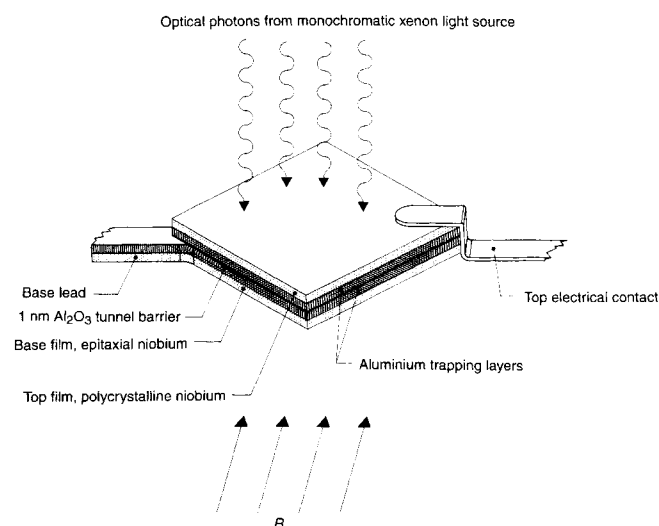


FIG. 1 A diagram of the $20 \times 20 \mu\text{m}$ device (not to scale). It was fabricated from a 'sandwich' of Nb/Al/ Al_2O_3 /Al/Nb multilayer deposited on polished sapphire. The base and top niobium films have a thickness of 100 nm; both the aluminium films are 120 nm thick. The insulating tunnel barrier of the device is highly transmissive with a resistivity of $2.2 \times 10^{-6} \Omega \text{cm}^2$. The sub-gap current density and dynamic resistance at the bias voltage of $V_b \approx 0.20$ mV were $\sim 2 \text{ pA } \mu\text{m}^{-2}$ and $\sim 0.3 \text{ M}\Omega$, respectively.

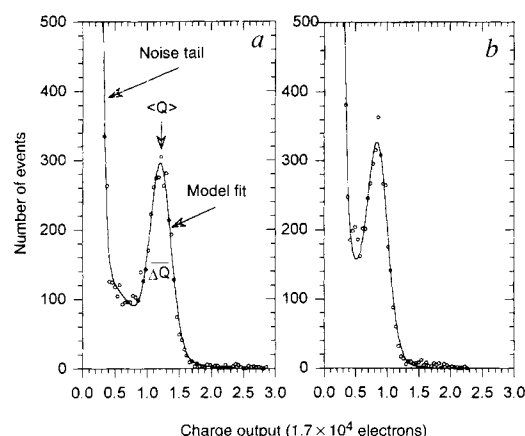


FIG. 2 The single photon charge spectrum (a histogram of the number of events giving a charge output Q from the detector) of the device when illuminated by a monochromatic source of photons of wavelength 250 nm (a) and 350 nm (b). The best-fit model spectra are also shown together with the definition of $\langle Q \rangle$ and $\Delta Q \approx 2.355 \sigma Q$ assuming a normal distribution.

The superconducting tunnel junction (STJ) has up until now been extensively investigated as a photon detector in the X-ray region of the spectrum where the number of free charge carriers generated after photoabsorption is $\sim 10^3$ times larger than in the optical region⁸⁻¹². A detailed review of the properties of such detectors and their performance at X-ray wavelengths can be found in ref. 12. Theoretical work has identified the potential use of STJs as optical photon counting detectors with high quantum efficiency and an inherent spectroscopic capability^{13,14}. To date, however, only limited experimental investigations of the response of superconductors, and in particular STJs, to optical wavelengths have been made¹⁵⁻¹⁷.

The STJ used in the present investigation is shown schematically in Fig. 1 and has been specifically designed to be extremely sensitive to single optical photons. The absorption of such a photon of wavelength λ in the top superconducting film (niobium in the present device) is followed by a series of processes in which the photon energy is converted into broken Cooper pairs and phonons¹⁰. This conversion process is very fast in niobium (~ 2 ns), after which an initial non-equilibrium distribution of both free charge carriers and phonons exist. At the very low operating temperature (typically about an order of magnitude lower than the superconductor's critical temperature T_c) the initial number of free charge carriers N_0 created as a result of the absorption of a photon can be significantly in excess of any thermally induced population. It can be shown¹⁰ that in general $N_0(\lambda) \approx h\nu/1.75\Delta$, and in the case of a niobium-based detector $N_0(\lambda) \approx 7 \times 10^5/\lambda\Delta$. Here Δ (in meV) is the temperature-dependent energy gap of the superconducting niobium, $h\nu$ is the photon energy, and λ is in nm. The energy gap separates the ground state of bound conduction electron pairs (Cooper pairs) from free electron excited states. A small magnetic field (B) is applied parallel to the barrier, as shown in Fig. 1, to suppress the tunnelling of Cooper pairs. Applying a bias voltage $V_b < 2\Delta/e$ ensures that the only tunnel processes which can occur involve the transfer of charge carriers from one film to the other and which therefore give rise to an electrical current. The oxide barrier is extremely thin (~ 1 nm) thereby ensuring a high transmission across the tunnel barrier. The aluminium films either side of the barrier act as a trap confining the charge carriers close to the barrier, leading to multi-tunnelling and maximizing the mean number of electron transfers $\langle n \rangle$ across the barrier¹⁸⁻²⁰. This process can be viewed as essentially an amplification of the original charge N_0 . It can be assumed that the trapping mechanism

is sufficiently fast to collect all the charge carriers N_0 that are created in the initial photoabsorption process in the top superconducting film.

A high-purity ultraviolet-grade silica fibre coupled to a xenon arc lamp light source was used to illuminate the device which was maintained at a temperature of 370 mK by means of a ^3He cryostat¹⁷. A monochromator, having a typical bandpass of ~ 10 nm, allowed the junction response to be studied at different wavelengths λ from ~ 220 to 500 nm. Although the complete system was calibrated at room temperature, an important reference calibration was also performed *in situ* at ~ 370 mK using a radioactive ^{55}Fe X-ray source which emits photons at a wavelength of ~ 0.2 nm. This calibration therefore allowed an estimate of $\langle n \rangle \approx 20$.

The device was initially illuminated with light at a very low intensity level such that only a single photon was incident on the detector over a period far larger on average than the charge carrier lifetime in the device ($\sim 2 \mu\text{s}$). Figure 2 illustrates the resulting single photon charge spectra Q from 250- and 350-nm photons. From these data it is clear that single photons can not only be detected but their wavelength measured. We measured a value of $\langle n \rangle$ of ~ 6 —a factor of ~ 3 less than for the X-ray case. This value, given the system noise from the room-temperature electronics, means that the detector's long-wavelength limit for single photon counting is currently ~ 550 nm. The single photon charge spectra, such as shown in Fig. 2, were fitted with a simple gaussian having the intensity, mean charge $\langle Q(\lambda) \rangle$ and standard deviation of the charge distribution σQ as free parameters, plus a noise component providing an additional gaussian tail at low charge values. Figure 3 illustrates the mean charge output $\langle Q(\lambda) \rangle$ versus λ^{-1} derived by this fitting procedure. The wavelength resolution of the detector can be determined from σQ (which is dominated by a measured electronics noise component σQ_{noise}) and was found to be a linear function of $\langle Q \rangle$. Such a relationship also exists at X-ray wavelengths in simple niobium devices and was attributed to an additional variance arising from a spatial variation in the response²¹. Figure 4 illustrates the variation with λ of the measured resolution $d\lambda_M$ (which for a gaussian response, such as is clearly shown in Fig. 2, can be simply defined as $d\lambda_M \approx 2.355 \lambda \sigma Q \langle Q \rangle^{-1}$). The best-fit model, which assumed the measured linear functions of σQ and λ with $\langle Q \rangle$, is also shown. A resolution of $\sim 37\%$ was therefore measured at a wavelength of 350 nm. The theoretical resolution is, however, significantly better

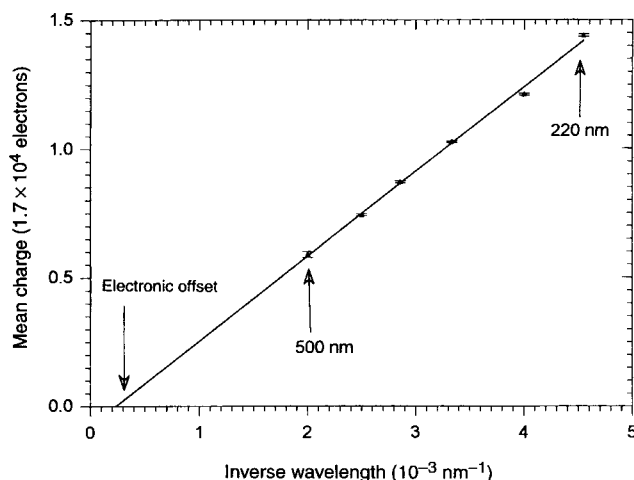


FIG. 3 The linearity of the detector when illuminated by single monochromatic photons as shown by a plot of the mean charge $\langle Q \rangle$ against λ^{-1} . The best-fit linear model is also indicated. The mean charge $\langle Q \rangle$ is linear with photon energy to within 2% of the fit of the form: $\langle Q(\lambda) \rangle = m_1 \lambda^{-1} + c_1$. In this fit, $m_1 = (5.66 \pm 0.15) \times 10^6$ electrons nm^{-1} and $c_1 = -1.3 \times 10^3$ electrons, which simply reflects a known calibrated offset in the electronics.

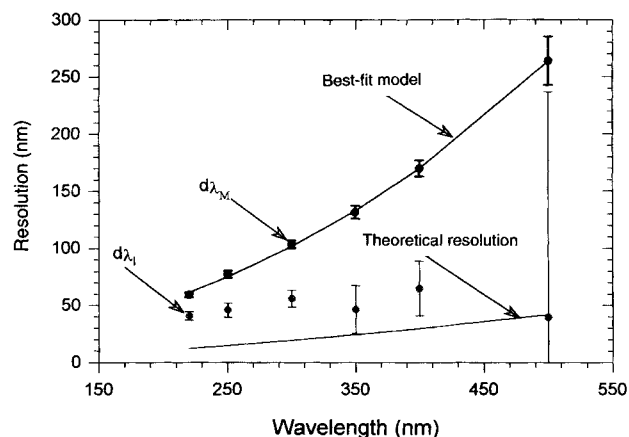


FIG. 4 The measured resolution $d\lambda_M$ of the device for single photons as a function of photon wavelength λ . Because $\langle Q \rangle = m_1/\lambda + c_1$ and $\sigma Q = m_2 \langle Q \rangle + c_2$, the measured resolution $d\lambda_M$ can be written as: $d\lambda_M = 2.355[m_2 m_1 \lambda + \lambda^2(m_2 + c_2)]/(m_1 + c_1 \lambda)$. The best-fit model is shown for $m_2 = 0.044$ and $c_2 = 1.8 \times 10^3$ electrons. The theoretical resolution of this specific device geometry is of the form $d\lambda(\text{nm}) \approx 2.8 \cdot 10^{-3} \lambda^{3/2} \Delta^{1/2} [F + 1 + (1/\langle n \rangle)]^{1/2}$, where F is the Fano factor which for niobium is ~ 0.22 and Δ is the superconducting niobium energy gap¹⁰. This theoretical resolution is shown for the case when $\langle n \rangle \approx 6$ together with the derived device limiting resolution $d\lambda_i$ after removal of the measured component of the electronic noise.

than this value and is based on the Fano and tunnel noise statistics (see Fig. 4 legend). The theoretical resolution, also shown in Fig. 4, is $\sim 7\%$ for 350-nm photons^{22,23}. Although the electronic noise, as determined from the pulsed stimulation of the preamplifier, dominates the measured resolution ($\sim 35\%$ at 350 nm), a perceptible dependence on wavelength of the actual device resolution can be discerned. Note that, in the present experimental configuration, the preamplifier is operated at room temperature over one metre from the device. Figure 4 shows the calculated device-limited resolution $d\lambda_i$ under the assumption that $\sigma Q_i^2 \approx \sigma Q^2 - \sigma Q_{\text{noise}}^2$. Although the errors are large, it is clear that the limiting resolution of this specific detector is $d\lambda_i \approx 45$ nm ($\sim 13\%$) at a wavelength $\lambda \approx 350$ nm.

Even the modest wavelength resolution reported here (~ 45 nm), which is some way from that theoretically achievable (~ 20 nm at $\lambda \approx 350$ nm), is close to that needed to provide an order-separation facility for high-resolution echelle spectrographs, which would obviate the need for a cross-dispersing element. Expected advances in the energy resolving capabilities would lead to a device that could be used for faint-object detection and spectroscopy (analogous to objective-prism type capabilities), to line imaging capabilities, and to broad- to intermediate-band (simultaneous) photometry.

Recording the arrival time of individual photons has many potential astronomical applications: (1) sub-millisecond capabilities could be applied to all aspects of atmosphere-limited observations (image sharpening, speckle imaging/spectroscopy, and adaptive optics applications, leading to improved spatial resolution and improved signal-to-noise ratios); (2) the timing capabilities, coupled with panoramic imaging, would be useful for applications related to object variability on short timescales (for example, pulsars, radial oscillations in white dwarfs and instabilities in accretion flows) and to a variety of unexplored areas of astronomical quantum optics²⁴; (3) applications such as space interferometry where an efficient, broad-band, photon-counting system is demanded.

Our experiments demonstrate that a niobium-based STJ can be used as a photon counting ultraviolet and optical detector with high dynamic range and with an inherent spectroscopic capability of ~ 45 nm at 350 nm. Further reductions in the electronic noise,

when coupled to improvements in the response uniformity of the detector, should allow the limiting theoretical resolution of ~ 20 nm at 350 nm (along with photon counting out to $1\text{ }\mu\text{m}$ or beyond) to be achieved. The device's inherent spectral resolution could be improved by using a superconductor with a lower critical temperature, for example aluminium, which would provide a limiting theoretical resolution of 8 nm at 350 nm. The present device is extremely efficient ($\sim 50\%$ over the waveband 200–1,000 nm), based on the film thickness and the optical constants of niobium²⁵. This absorption efficiency could potentially be further increased through the introduction of anti-reflection coatings. These detectors are also intrinsically very fast, being limited primarily by the event signal processing electronics. A typical

speed of ~ 10 kHz has already been demonstrated in pure niobium dominated devices at photon wavelengths of ~ 1 nm; higher rates may be possible²⁶. The device reported here has been operated at ~ 2.5 kHz without any discernible degradation in performance. If a large-format array can be fabricated together with its associated preamplifiers, then (notwithstanding the need for operation below ~ 1 K through the use of a liquid-helium-based cryostat) such a high-efficiency spectrophotometric detector could become an important instrument for astronomical observations at optical and ultraviolet wavelengths.

Note added in proof: Recent improvements in the response of the electronics have lowered the low-frequency cut-off of the detector to over $1\text{ }\mu\text{m}$. $\square$

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- Jacoby, G. H. in *CCD's in Astronomy* **424**, (Conf. Ser. 8, Astr. Soc. Pacif., 1990).
- Janesick, J. et al. *Proc. SPIE* **2231**, 223–237 (1990).
- Delamere, A. in *Photon Detectors for Instrumentation* 111–113 (SP-356, ESA, Noordwijk, 1992).
- Boksenberg, A. in *Image Processing Techniques in Astronomy* **15**, (eds de Jager, C. & Nieuwenhuizen) (Reidel, Dordrecht, 1975).
- Cullum, M. in *Instrumentation for Ground-Based Optical Astronomy, Present and Future* (ed. Robinson, L. B.) 511–515 (Springer, New York, 1988).
- Timothy, J. in *Instrumentation for Ground-Based Optical Astronomy, Present and Future* (ed. Robinson, L. B.) 516–519 (Springer, New York, 1988).
- Petroff, M. D. & Stapelbroeck, M. G. *IEEE Trans. Nucl. Sci.* **36**, 158–162 (1989).
- Wood, G. H. & White, B. *Appl. Phys. Lett.* **15**, 237–238 (1969).
- Twerenbold, D. *Europhys. Lett.* **1**, 209–210 (1986).
- Rando, N. et al. *Nucl. Instrum. Meth.* **A313**, 173–195 (1992).
- Twerenbold, D. *Phys. Rev.* **B34**, 7748–7759 (1986).
- de Korte, P. in *Photon Detectors for Instrumentation* (SP-356, ESA, Noordwijk, 1992).
- Perryman, M. A. C., Foden, C. L. & Peacock, A. in *Photon Detectors for Instrumentation* 21–26 (SP-356, ESA, Noordwijk, 1992).

- Perryman, M. A. C., Foden, C. L. & Peacock, A. *Nucl. Instrum. Meth.* **A325**, 319–325 (1993).
- Chi, C. C. et al. *Phys. Rev.* **B23**, 124–132 (1981).
- Kurakado, M. & Matsumura, A. in *Int. Superconductivity Conf. ISEC* Vol. 89, 59–62 (Tsukuba, Ibaraki 305, Japan, 1989).
- Rando, N. et al. *J. low temp. Phys.* **93**, 659–664 (1993).
- Gray, K. *Appl. Phys. Lett.* **32**, 392–395 (1978).
- Booth, N. *Appl. Phys. Lett.* **50**, 293–295 (1987).
- Mears, C. A., Labov, S. E. & Barfknecht, A. T. *Appl. Phys. Lett.* **63**, 2961–2963 (1993).
- Verhoeve, P. et al. *Phys. Rev. B* (in the press).
- Fano, U. *Phys. Rev.* **72**, 26–29 (1947).
- Goldie, D. J. et al. *Appl. Phys. Lett.* **64**, 3169–3171 (1994).
- Dravins, D. *ESO Messenger* Vol. 78, 9–19 (ESO, München, 1994).
- Weaver, J. H. et al. *Physik Daten, Fach-informations zentrum* Nr. 18-1, 1–71 (Haslab, Desy, Hamburg, 1981).
- Lumb, D. et al. *Proc. SPIE* **2518**, 258–267 (1995).

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A universal scaling law for atomic diffusion in condensed matter

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THERE is currently no unifying quantitative description of atomic diffusion in condensed matter. Analytic expressions have been obtained for the transport coefficients of an idealized dense fluid of hard spheres^{1,2}, but their generalization to the rich variety of atomic structures in real condensed systems remains a challenge. Here I present evidence from molecular dynamics simulations that a universal relationship exists between the structure and the equilibrium rate of atomic diffusion in liquids and solids. I find that the diffusion coefficient, reduced to a dimensionless form by scaling by the atomic collision frequency and the atomic diameter, is uniquely defined by the excess entropy, a measure of the number of accessible configurations of the system. A scaling law relating these two quantities holds well for simple liquids, and also remains applicable to atomic transport in a quasicrystal and to silver-ion diffusion in the solid-state ionic conductor α -AgI. This makes it possible to estimate diffusion coefficients directly from diffraction measurements of an equilibrium structural characteristic, namely the radial distribution function of the diffusing species.

Atomic dynamics in condensed phases is dominated by the so-called cage effect², whereby the diffusive atomic motions are coupled to the structural relaxations. Owing to the pronounced structural correlations, each atom finds itself in a cage formed by the immediate neighbours, and its diffusive motion is effected by a local density fluctuation transforming the surrounding atomic configuration. The kinetic theory expressions describing this

diffusion mechanism^{3,4} represent a generalization of the Enskog theory⁵ for a dense hard-sphere fluid; to apply this description to a real system, a hard-sphere model is required. This approach is based on the assumption that the local order, which controls the rate of the cage diffusion, is induced by the effect of impenetrability of the atomic hard cores, and is insensitive to the longer-ranged interaction between atoms. But the structural diversity of condensed atomic phases, and the dependence of this diversity on the details of interatomic interactions, impose a fundamental limitation on the range over which the hard spheres can adequately describe real systems.

The approach suggested here includes the following two arguments. First, the transfer of momentum and energy at high densities is facilitated by the short-range repulsive interatomic interactions, which can be described as binary collisions of hard spheres. The rate of this process is thus defined by the collision frequency which, according to the Enskog theory⁵, can be calculated for the temperature T and the number density ρ as:

$$\Gamma_E = 4\sigma^2 g(\sigma) \rho \sqrt{\pi k_B T / m} \quad (1)$$

where m and σ are the atomic mass and the hard-sphere diameter, respectively; $g(\sigma)$, the value of the radial distribution function at the contact distance, represents the density of the immediate neighbours. In a real system, σ can be interpreted as the position of the first maximum of $g(r)$. Using σ and Γ_E^{-1} as natural units of length and time, respectively, the diffusion coefficient D can be expressed in a dimensionless form as $D^* = D \Gamma_E^{-1} \sigma^{-2}$.

The second argument concerns the general relationship between the structure and atomic transport in a condensed system. The frequency of the local structural relaxations, which defines the rate of the cage diffusion, is obviously proportional to the number of accessible configurations (per atom). In an equilibrium system, the constraints imposed by the structural correlations reduce this number by a factor of e^S , where S is the excess entropy, the difference between the systems thermodynamic entropy and that of the equivalent ideal gas; hence, e^S and D^* must be connected by a universal linear relationship. Furthermore, it

is assumed here that S can be restricted to the two-particle approximation^{6,7}:

$$S_2 = -2\pi\rho \int_0^\infty \{g(r)\ln[g(r)] - [g(r) - 1]\}r^2 dr \quad (2)$$

To test this conjecture, a diverse set of simple monatomic liquids was simulated by molecular dynamics. The simulation utilized the pair potentials, presented in reduced units in Fig. 1a. These pair potentials induce three distinct prototypes of liquid structure, with contrasting radial distribution functions (Fig. 1b). The structure of the well known Lennard-Jones (LJ) liquid, related to the face-centred cubic pattern, can be approximated by hard spheres⁸. Another liquid (IC) is characterized by predominantly icosahedral local order⁹, while in the third liquid model (HX) the local order is topologically related to the primitive hexagonal lattice¹⁰ with only six nearest neighbours (note its anomalously small $g(\sigma)$ in Fig. 1b). The Arrhenius plot for constant-density runs (Fig. 1c) demonstrates dramatic distinctions between the atomic diffusivities of these liquids.

Diffusion variation in LJ liquid was also investigated at constant pressure. Other monatomic liquid models examined here include two liquid metals, Cu (ref. 11) and Pb (ref. 12), as well as the hard-sphere fluid. To avoid possible size effects, the simulation employed a system of 16,384 particles.

The results are shown in Fig. 2a, and demonstrate convincingly that there exists a universal relationship between D^* and S_2 . This observation implies that atomic diffusion is an entirely geometrical phenomenon which can be uniquely and universally accounted for by the frequency of binary collisions and the excess entropy, representing the measure of structural uncertainty. Moreover, it is found that throughout the range of S_2 that corresponds to the liquid domain, the results can be described by a

simple scaling law:

$$D^* = 0.049e^{S_2} \quad (3)$$

which is consistent with the conjecture formulated above.

In the classical Enskog theory, describing the dynamics of dilute gases in terms of uncorrelated binary collisions of hard spheres, the structural effects, which enter equation (1) as $g(\sigma)$, are confined to the first neighbour shell. In the cage diffusion model, fluctuations in the first shell of neighbours, facilitating the diffusive motions, are constrained by the structural correlations at larger distances. These correlations can thus be regarded as a distinction between liquids, dominated by the cage diffusion, and gases. Indeed, it is found that the contribution to S_2 from the part of $g(r)$ beyond its first peak becomes negligible for $-S_2 < 2$, where the Enskog results converge with equation (3) (Fig. 2a). At the same time, the simulation results for these values of S_2 develop a positive non-Enskog component. This manifests a crossover from cage diffusion to vortex diffusion¹³, observed in moderately dense gases, whereby the diffusive motions are enhanced by the coupling to the viscous modes.

The results presented in Fig. 2b demonstrate that equation (3) is not confined to the domain of conventional liquid dynamics. One case examined here is IC liquid near its melting point, where a high rate of vacancy-driven activated hopping was observed¹⁴. Another non-conventional transport phenomenon shown in Fig. 2b is atomic diffusion in an equilibrium quasicrystal, recently observed in a molecular dynamics simulation^{15,16}; this process is driven by a generic form of structural relaxation associated with incommensurate degrees of freedom. In spite of the fact that these two diffusion mechanisms are profoundly different from conventional liquid dynamics, they can be described by equation (3) with the same degree of accuracy as the liquid diffusion.

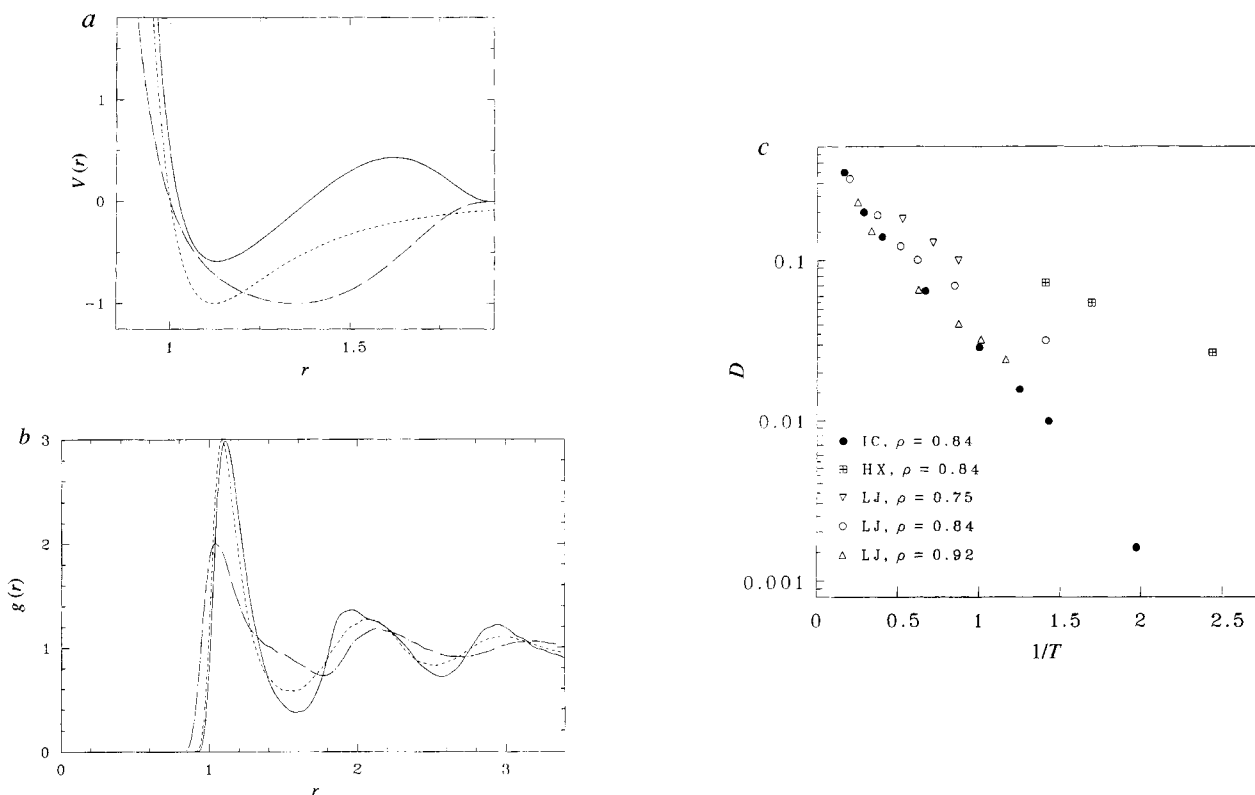


FIG. 1 Three liquid models examined in the molecular dynamics simulation. a, The pair potentials, expressed in the reduced units; b, the respective radial distribution functions, as calculated at the reduced density $\rho = 0.84$ and the temperature $T = 0.7$. Dashed line, the Lennard-Jones system (LJ); its local order is related to the face-centred cubic lattice. Solid line, IC liquid, possessing a predominantly icosahedral local order⁹; note the pronounced

structuring of its $g(r)$. Dot-dashed line, HX model; its local order is topologically related to the primitive hexagonal lattice with six nearest neighbours¹⁰, which is manifested by the anomalously small first peak of its $g(r)$. c, Arrhenius plot for constant-density diffusion variations in IC, HX and LJ liquids.

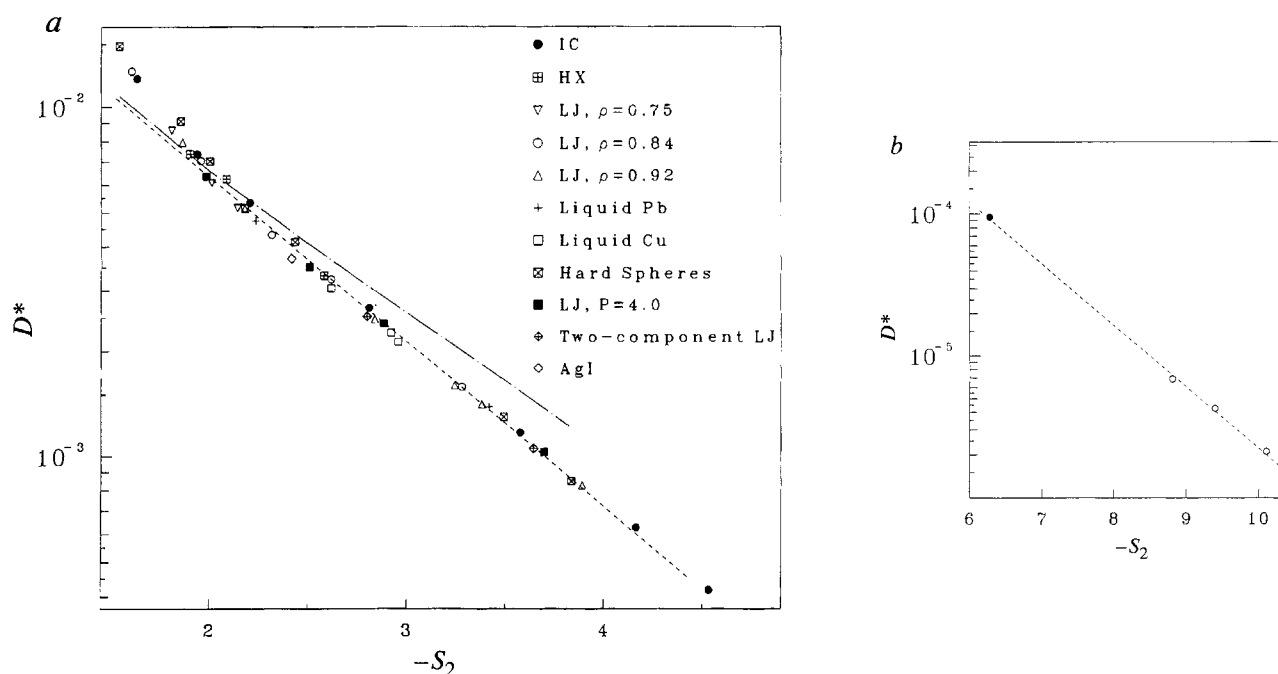


FIG. 2 a, The diffusion coefficient D^* for various models, as a function of the excess entropy, in the pair approximation, equation (2). D^* is dimensionless, scaled by $\Gamma_E^{-1}\sigma^{-2}$, where Γ_E is the Enskog collision frequency⁵ defined by equation (1), the effective hard-sphere diameter σ is estimated as the position of the first peak of $g(r)$. The plotted data for LJ, IC and HX models are those shown in Fig. 1c. For LJ liquid, isobaric diffusion variation $P = 4.0$ is also included. Liquid Cu (ref. 11) and liquid Pb (ref. 12) were simulated at observable conditions. Two partial diffusion coefficients are shown for a two-component LJ liquid model, simulated at $\rho = 0.84$ and $T = 0.7$. The two

constituent types of atoms have equal number densities and the diameter ratio of 1.25. The result for Ag diffusion in solid AgI at 804 K was obtained on a molecular-dynamics model¹⁷ based on pair interatomic potentials¹⁸. Dashed line, equation (3); dot-dashed line, classical Enskog theory results for hard spheres⁵. b, D^* as a function of S_2 for two cases of non-conventional diffusion: filled circle, IC liquid at the melting point, where a high rate of vacancy-induced hopping was observed¹⁴; open circles, a quasicrystal phase^{15,16}. The dashed line is the same as in a.

In a multicomponent system, Γ_E , σ and S_2 for each constituent type of atoms can be derived from the relevant partial $g(r)$ using equation (1) and (2). One test case presented in Fig. 2a is an LJ liquid composed of two kinds of atoms with equal number densities and the ratio of atomic diameters 1.25; for both components, good agreement with equation (3) is observed. Another simple case of diffusion in a binary system tested here is a molecular dynamics model¹⁷ of solid α -AgI at 804 K, based on pair interionic potentials¹⁸. Its two sub-systems are decoupled: I^- ions form a lattice, whereas diffusing Ag^+ ions are distributed randomly over available sites. Consistency of this diffusion process with the scaling law of equation (3) is demonstrated in Fig. 2a.

A tentative conclusion is that the observed relationship between D and $g(r)$, as expressed by equations (1), (2) and (3), is universal for equilibrium condensed atomic systems, both liquid and solid, regardless of the structures, interatomic interaction potentials or the microscopic dynamical mechanisms involved. D can thus be

calculated from the diffraction data in those cases where its direct measurement is not possible, or inferred from a structural model. The above results indicate that this scaling law can be applied to ionic conducting glasses¹⁹. A more general conclusion is that the rate of exploration of the configurational space, which, in condensed systems, defines the diffusion rate, is controlled by the entropy. This conclusion is consistent with the recent finding that the transition to a phase with lower entropy, observed in some liquids^{20–22}, results in a dramatic change in the rheological behaviour²³. I note that an attempt to relate the relaxation rate and the entropy was made 30 years ago by Adam and Gibbs²⁴; their conjecture, however, involved TS rather than S alone. In the systems which are non-ergodic, that is which cannot explore the entire region of the configurational space as defined by the structure, a negative deviation from equation (3) would be observed. In fact, such a deviation can be regarded as a quantitative criterion of non-ergodicity, giving an insight into the kinetics of glass formation. □

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- Boon, J. P. & Yip, S. *Molecular Hydrodynamics* (McGraw-Hill, New York, 1980).
- Cohen, E. D. G. *Physica A* **194**, 229–257 (1993).
- Cohen, E. D. G. & de Schepper, I. M. J. *statist. Phys.* **63**, 241–248 (1991).
- Kirkpatrick, T. R. & Nieuwoudt, J. C. *Phys. Rev. A* **33**, 2658–2662 (1986).
- Chapman, S. & Cowling, T. G. *The Mathematical Theory of Non-uniform Gases* (Cambridge Univ. Press, 1939).
- Baranyai, A. & Evans, D. *Phys. Rev. A* **40**, 3817–3822 (1989).
- Mountain, R. D. & Raveche, H. J. *chem. Phys.* **35**, 2250–2255 (1971).
- Hansen, J.-P. & McDonald, R. *Theory of Simple Liquids* (Academic, London, 1976).
- Dzugutov, M. *Phys. Rev. A* **46**, 2924–2927 (1992).
- Stillinger, F. & La Violette, R. A. J. *chem. Phys.* **83**, 6413–6418 (1985).
- Dzugutov, M., Alvarez, M. & Lomba, E. J. *Phys.: Condens. Matter* **6**, 4419–4428 (1994).
- Dzugutov, M., Larsson, K.-E. & Ebbsjö, I. *Phys. Rev. A* **38**, 3609–3618 (1988).
- Cucier, R. I. & Mehaffey, J. R. *Phys. Rev. B* **18**, 1202–1213 (1978).
- Dzugutov, M. *Europhys. Lett.* **26**, 533–538 (1994).

- Dzugutov, M. *Phys. Rev. Lett.* **70**, 2924–2927 (1993).
- Dzugutov, M. *Europhys. Lett.* **31**, 95–100 (1995).
- McGreevy, R., Chahid, A. & Ebbsjö, I. in *Annual Report 1995* (Studsvik Neutron Research Lab., Nyköping, Sweden, 1996).
- Vashista, P. & Rahman, A. *Phys. Rev. Lett.* **40**, 1337–1340 (1978).
- Angell, C. A. *Chem. Rev.* **90**, 523–542 (1990).
- Poole, P. H., Sciortino, F., Essmann, U. & Stanley, H. E. *Nature* **360**, 324–328 (1992).
- Aasland, S. & McMillan, P. F. *Nature* **369**, 633–636 (1994).
- Poole, P. H., Sciortino, F., Grande, T., Stanley, H. E. & Angell, C. A. *Phys. Rev. Lett.* **73**, 1632–1635 (1994).
- Angell, C. A. *J. phys. Chem.* **97**, 6339–6342 (1993).
- Adam, G. & Gibbs, J. H. *J. chem. Phys.* **43**, 139–146 (1965).

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Low-threshold cold cathodes made of nitrogen-doped chemical-vapour-deposited diamond

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BECAUSE diamond surfaces terminated with hydrogen have a negative electron affinity¹⁻⁴ (the conduction band minimum lies below the vacuum level), they are expected to emit electrons spontaneously. This has led to attempts to develop 'cold cathodes'—miniaturized vacuum diodes that might have applications in microelectronics and flat-panel displays. In previous studies of electron emission from diamond grown by chemical vapour deposition⁵⁻⁹ (CVD), the threshold voltages for emission were more than an order of magnitude too large for use in battery-driven cold cathodes. Although low-threshold emission from caesium-coated, nitrogen-doped high-pressure synthetic diamond was reported recently¹⁰, ultimately diamond thin films grown by chemical vapour deposition (CVD) look to be the most promising material for cold-cathode applications. But to obtain low-threshold emission, it is necessary to introduce high concentrations of donor dopants such as nitrogen—something that is difficult for CVD diamond. Here we report that high concentrations of nitrogen can be incorporated into diamond films by using urea as the gaseous nitrogen source, and that such heavily doped films shown very-low-threshold electron emission, which augurs well for cold-cathode technology.

Doped diamond films were grown using the hot-filament CVD technique under an atmosphere of acetone and hydrogen¹¹. The growth conditions were as follows; the tungsten filament temperature was 2,300 °C, the substrate temperature was 850 °C, the reaction pressure was 100 torr and the ratio of reactant gas to hydrogen was 0.6%. A saturated solution of urea ((NH₂)₂CO) and

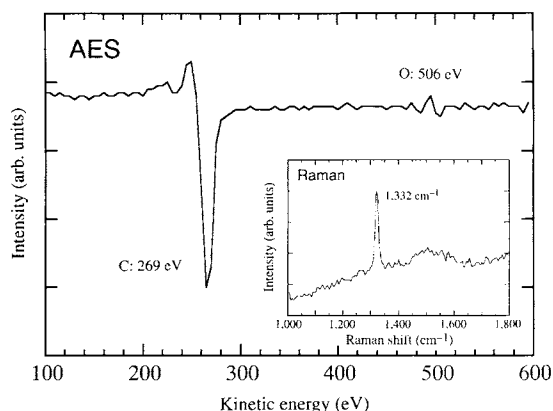


FIG. 1 Auger electron spectrum (AES; main figure) and Raman spectrum (inset) of nitrogen-doped diamond using urea as the dopant. See text for discussion of the peaks in these spectra.

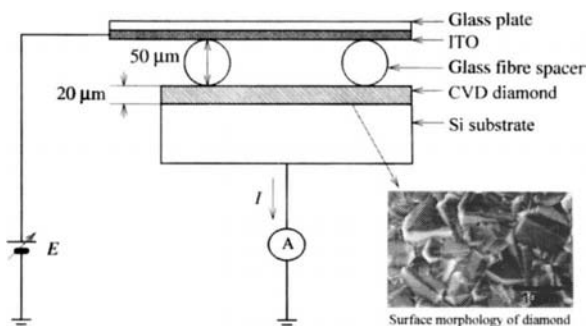


FIG. 2 Main figure, schematic structure of the electron-emission measurement circuit; inset, morphology of the diamond surface doped with nitrogen. The base pressure of the vacuum system is 5×10^{-7} torr and the leakage current of the system is less than 2×10^{-9} A.

methanol (CH₃OH) was diluted to 1/10 with acetone ((CH₃)₂CO), and vaporized to be used as the reactant gas. The films were grown for 2 hours on 2.5×2.5 mm n-type (100)Si substrates; the surfaces of these substrates had been scratched with diamond paste (grain size, 1 μm) to give a high enough nucleation density for growth of a continuous film.

Films were characterized both on their surfaces and in the bulk using electron diffraction, Auger electron spectroscopy (AES) and Raman spectroscopy. In the AES spectrum shown in Fig. 1, there is a peak due to carbon around 269 eV and an oxygen peak around 506 eV, suggesting that a small number of oxygen atoms (as well as carbon atoms) exist on the surface. The interplanar spacing obtained from the electron-diffraction pattern agreed with that expected from polycrystalline diamond. In addition, the strong peak at $1,332 \text{ cm}^{-1}$ in the Raman spectrum (Fig. 1 inset) confirms that the bulk of the film is diamond containing only small amounts of amorphous and graphitic components.

The concentration of nitrogen in the diamond film was evaluated using the Rutherford back-scattering (RBS) technique. The RBS data reveal the N concentration in the diamond films to be $0.2 \pm 0.06\%$, equivalent to $\sim 3.5 \times 10^{20}$ N atoms per cm^3 . Such a high concentration of N is usually difficult to incorporate in diamond when using nitrogen (N₂) or ammonia (NH₃) as the dopants (J. C. Twichell and M. W. Geis, personal communication), and it seems that this heavy doping of N is achieved only when (NH₂)₂CO is used as the dopant. Although there is a possibility of N segregation both in the crystallites and in the grain boundaries, it is clear that in terms of overall electron emission properties, incorporation of N into polycrystalline CVD-grown diamond leads to the dramatic improvements discussed below.

The sample was mounted as the cathode in a vacuum system with a base pressure of $\sim 5 \times 10^{-7}$ torr. A glass plate coated with indium tin oxide (ITO) was placed 50 μm above the cathode as the anode using glass-fibre spacers, and the emission current (I) against the anode voltage (V) was measured (Fig. 2). The leakage current in this measuring system was confirmed to be less than 2×10^{-9} A, which is the detection limit, from the fact that there was no current observed when the applied voltage was reversed, or when a molybdenum plate was mounted instead of diamond cathodes. The electric field (F) was calculated by dividing the anode voltage by the anode-cathode distance, and the current density (J) was obtained assuming that the emission occurred over the entire surface of the film. This assumption gives only the minimum current density; the emission is more likely to be occurring at particular sites rather than over the entire surface, and therefore a higher local current density must be expected. The surface morphology of the film observed by the scanning electron microscope (SEM; Fig. 2 inset) indicated that the film surface is not flat and the size of each crystallite is 8 μm on average.

Remarkable J - F characteristics of the N-doped diamond are

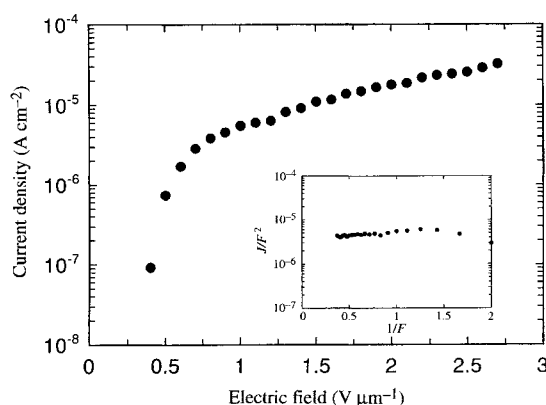


FIG. 3 Main figure, plot of emission current density (J) against electric field (F) characteristics of nitrogen-doped diamond. The threshold voltage is observed as low as $0.5 \text{ V } \mu\text{m}^{-1}$. Note that no linear relationship is observed in the J/F^2 versus $1/F$ plot (Fowler-Nordheim characteristics) inset.

shown in Fig. 3, indicating that the threshold field is less than $0.5 \text{ V } \mu\text{m}^{-1}$. When the distance between the cathode and the anode was changed, almost the same J - F characteristics were observed indicating that there is a linear relation between anode voltage and distance. Furthermore, no linear relation was observed in the J/F^2 versus $1/F$ characteristics (Fig. 3 inset) suggesting that the current limit is not dominated by the standard tunnelling theory proposed by Fowler and Nordheim¹². Although the total current density integrated over the entire anode area seems to be too low for the space-charge-limited current (SCLC) mechanism, if a much higher local current density occurs at the cathode, the current could be limited by the SCLC mechanism. In general, if the slope n of the J - F characteristics ($J = kF^n$) is 1.5, this confirms the Langmuir-Child law suggesting that the current is limited by SCLC in vacuum; if $n = 2$, the current is dominated by the SCLC in the diamond bulk. The value of n obtained from Fig. 3 is 1.75, and it is difficult at present to determine which mechanism is more probable. Defect/trap limited SCLC through the diamond is another possible reason for the current limitation, but the physical interpretation of this process still remains to be clarified.

Comparison of the emission properties of N-, phosphorus (P)- and boron (B)-doped diamond films are shown in Fig. 4. The surface morphologies of the P- and B-doped films were just the same as that of N-doped film shown in Fig. 2 inset^{11,13}, and there seems to be no large variation in the enhancement factor evaluated from the radius of curvature. This leads us to the view that

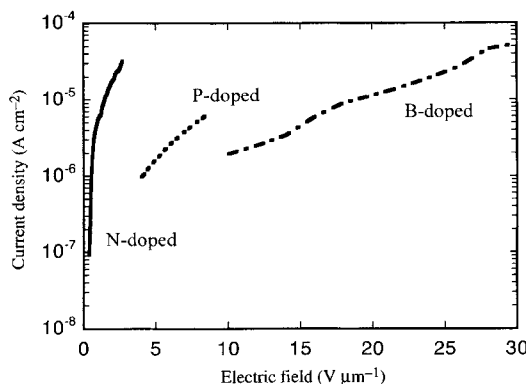


FIG. 4 Comparison of emission properties of nitrogen (N-), phosphorus (P)- and boron (B)-doped polycrystalline diamond films. The threshold field is much lower in the N-doped diamond film than P- and B-doped diamond films.

the difference in the emission properties must be fundamentally due to the difference in impurities introduced into the diamond films. It is clear from the figure that the threshold field is much lower in the N-doped sample than P- or B-doped samples, indicating that incorporation of N in diamond plays an important role in determining the emission characteristics.

As far as we know, such low-threshold emission has not previously been reported before for any type of diamond without caesium coating. If this material was used as the cathode in vacuum microelectronic devices, a single commercial 1.5 V battery would provide a current density of more than $10^{-6} \text{ A cm}^{-2}$ when the gap between the anode and the gate was $3 \mu\text{m}$ (a value which can be achieved readily with present technology). This will greatly simplify cathode switching as standard digital electronics could be used. Although bright luminescent displays with N-doped diamond cathodes can be realized with existing phosphors, with the achievement of such low electron-emission thresholds, the emphasis of research should be placed on obtaining stable phosphors that give enough brightness even when the electrons have significantly lower energies. □

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- Himpel, F. J., Knapp, J. A., VanVechten, J. A. & Eastman, D. E. *Phys. Rev.* **B20**, 624-627 (1979).
- Pate, B. B. *et al. J. Vac. Sci. Tech.* **19**, 349-354 (1981).
- Geis, M. W., Gregory, A. & Pate, B. B. *IEEE Trans. Electron Devices* **38**, 619-626 (1991).
- van der Weide, J. & Nemanich, R. J. *Appl. Phys. Lett.* **62**, 1878-1880 (1993).
- Wang, C., Garcia, A., Ingram, D. C., Lake, M. & Kordesch, M. E. *Electron. Lett.* **27**, 1459-1460 (1991).
- Xu, N. S., Latham, R. V. & Tzeng, Y. *Electron. Lett.* **29**, 1596-1597 (1993).
- Twitchell, J. C. *et al. (abstr.) Diamond Films '93* 17.1 (COMST, Switzerland, 1993).
- Okano, K., Hoshina, K., Iida, M., Koizumi, S. & Inuzuka, T. *Appl. Phys. Lett.* **64**, 2742-2744 (1994).
- Zhu, W. *et al. Appl. Phys. Lett.* **67**, 1157-1159 (1995).
- Geis, M. W., Twitchell, J. C., Macaulay, J. & Okano, K. *Appl. Phys. Lett.* **67**, 1328-1330 (1995).
- Okano, K. *et al. Jap. J. appl. Phys.* **27**, L173-L175 (1988).
- Fowler, R. H. & Nordheim, L. *Proc. R. Soc. Lond. A* **119**, 173-181 (1928).
- Okano, K. *et al. Appl. Phys.* **A51**, 344-346 (1990).

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Increased UV-B penetration in a lake owing to drought-induced acidification

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CLIMATE change, acid deposition and increasing solar ultraviolet irradiance¹ have all combined to produce marked effects on lake waters and their ecosystems. Schindler *et al.*² have shown that both climate warming and lake acidification have led to decreases in dissolved organic carbon concentrations in North American boreal lakes, resulting in a markedly increased exposure of the upper water column to solar ultraviolet radiation. Here we report ten years of observations of rainfall and lake chemistry at Swan Lake, Canada, that suggest that a fall in dissolved organic carbon

concentrations can also occur by a different combination of climate change and acidification. In this boreal fresh water, a drought decreased lakewater levels, thus exposing to the atmosphere littoral sediments containing reduced sulphur from the previous atmospheric deposition of industrial sulphur dioxide into the lake and its catchment. This exposure caused a re-oxidation of sediment sulphur, resulting in a remobilization of acid into the lake water, and thus a decrease in dissolved organic carbon concentrations sufficient to increase by three-fold the depth to which ultraviolet radiation can penetrate.

Swan Lake (46° 22' N, 81° 04' W) is a small (area, 5.8 ha; maximum depth, 8.8 m), rapidly flushing (water renewal time of < 1 yr) lake located on the Canadian Precambrian Shield 13 km from the smelting complexes in Sudbury, Canada. The lake has a sparsely forested, uninhabited catchment. Swan Lake is one of the thousands of Sudbury lakes³ that have been acidified by the atmospheric deposition of industrial SO₂ emissions. Palaeolimnological reconstruction indicates that Swan Lake began to acidify 50 years ago, reaching a minimum in pH in the 1970s⁴. The measured pH averaged 3.97 in 1977⁵. Additional palaeolimnological, chemical and biological descriptions of Swan Lake are provided elsewhere^{4,6-9}.

During the early 1980s, the water quality of many Sudbury

lakes, including Swan Lake, improved in response to reductions in emissions of SO₂ from local copper/nickel smelters¹⁰. The SO₄²⁻ concentration of Swan Lake was 580 µequiv. l⁻¹ in 1977⁵, but dropped to 170 µequiv. l⁻¹ by 1985 (Fig. 1b). Concurrently, pH increased by almost 2 to 5.8 (Fig. 1c). However, 1986 and 1987 were extremely dry (Fig. 1a); in response, the lake surface area shrank by 18%, exposing large areas of littoral sediments to the atmosphere. In 1988, the wettest year since 1960⁶, the water level rose, the SO₄²⁻ concentration in Swan Lake increased to 400 µequiv. l⁻¹ (Fig. 1b), and lake pH dropped to 4.5 (Fig. 1c). Oxidation of reduced sulphur compounds in the exposed littoral zone and elsewhere within the catchment during the drought, followed by flushing in the subsequent wet year, is the likely cause of the SO₄²⁻ increase and resulting re-acidification^{6,11}, as there was no concurrent increase in SO₂ emissions from the smelters⁶.

In regions where the available base-cation pool exceeds the pool of re-oxidizable sulphur, drought may not lead to acidification¹²; however, the response of pH and SO₄²⁻ in Swan Lake is not unique. Bodo and Dillon¹³ reported that the 20-yr reversal of acidification in Clearwater Lake, near Sudbury, was interrupted in 1977 and 1988, the only years preceded by two-year droughts. In their annual survey, Keller *et al.*⁶ noted that the pH of 31 of 38 Sudbury lakes dropped in 1988, for the first time since 1980. Similarly, Kelso *et al.*¹⁴ recorded in 1988 a drop in average pH in 56 headwater lakes in north-central Ontario, 275 km west of Sudbury.

Concurrent with its acidification, the concentration of dissolved organic carbon (DOC) in Swan Lake decreased from 190 µmol Cl⁻¹ in 1987 to 63 µmol Cl⁻¹ in 1988 (Fig. 1e). Declines in DOC are an expected consequence of acidification^{2,15-18}. Although several factors may have contributed to this particular decline, two are most significant. The DOC decline may be a direct result of acidification to pH < 5 (ref. 15). Increases in aluminum concentration, a common consequence of acidification^{19,20}, can also lower DOC concentrations. In Swan Lake, the increase in Al in 1988 (Fig. 1d) produced concentrations exceeding the 1.4–2.8 µmol total Al per mg of DOC needed to saturate and flocculate DOC originating in wetlands²¹, the major DOC source for lakes on the Precambrian Shield²².

Temporal changes in the diffuse vertical attenuation coefficient (*K_d*) of UV-B radiation in Swan Lake were estimated from the measured changes in DOC concentration (Fig. 1e) using an empirical model based on 16 North American lakes²³, including Swan Lake. The model inferred that the re-acidification of Swan Lake substantially increased UV-B penetration. The median modelled annual *K_d* decreased by three-fold between 1987 and 1988, from 2.54 to 0.86 m⁻¹. This corresponded to an increase in the depth of lake waters exposed to 1% of lake surface UV-B irradiance, from 1.8 m in 1987 to 5.6 m in 1988 (Fig. 1f), the latter corresponding to 94% of the lake's volume.

In Swan Lake, the change in DOC concentration was linked to climate through drought-induced acidification. In other lakes, more direct linkages are possible. The DOC concentration may decline during droughts in response to decreased DOC export from catchments, and/or more efficient removal from the water column over the longer water renewal times^{2,18} (Fig. 2).

Manabe and Wetherald²⁴ estimate that soil moisture content in eastern North America, the region of highest atmospheric sulphur deposition on the continent, will decline by 10–40% if atmospheric CO₂ concentrations double. One likely consequence of such reductions in soil moisture is an increase in the amount of strong acid exported from sulphur-enriched catchments¹¹. The size of the reservoir of reduced sulphur that would supply this acidity, a legacy of a century of elevated industrial SO₂ emissions²⁵, has not been quantified. If the drought-induced increase in export of acid from catchments exceeds the decrease attributable to the anticipated reductions in anthropogenic emissions of SO₂ (ref. 26), then many lakes will acidify. Rapidly flushing lakes that have wetlands in their catchments and are in high sulphur

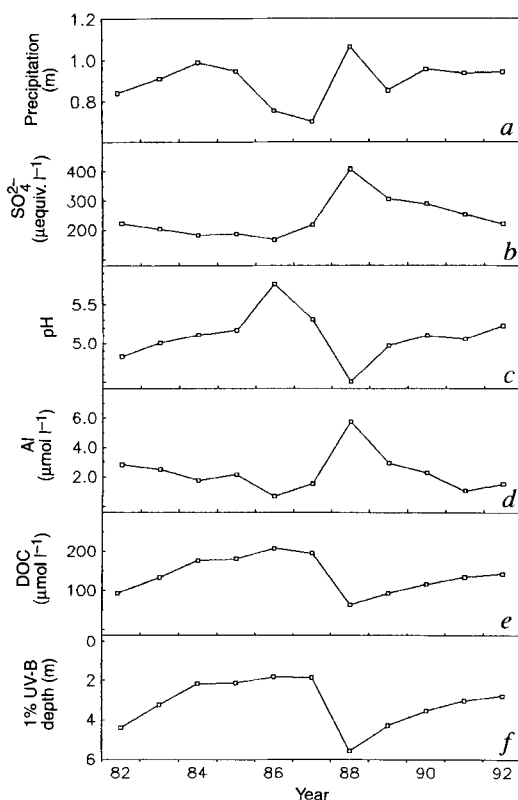
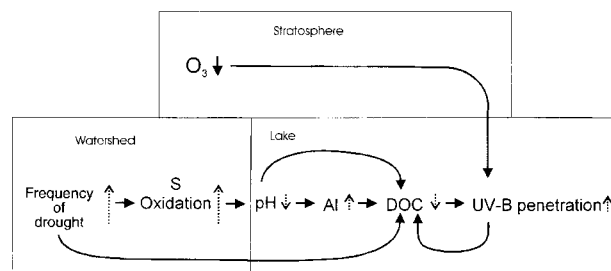


FIG. 1 a, Annual precipitation depths measured at the Sudbury airport. b–e, Measured quantities in Swan Lake as follows: b, sulphate concentration; c, pH; d, total Al; e, DOC concentrations. f, Calculated depth of 1% of surface UV-B irradiance. The 1% depth was calculated from the diffuse vertical attenuation coefficient (*K_d*) using the relation: $K_d = 0.60 + 0.389\text{DOC}^{1.873}$, $r^2 = 0.97$, which was constructed from the open-lake profiles data in ref. 23 (DOC in mg l⁻¹, as ref. 23). Chemical analyses were performed on bathymetrically weighted composite samples made from water collected at 1-m intervals through the water column. Composites were generated on alternate weeks throughout the seven-month ice-free season. DOC concentrations were determined by automated colorimetric titration of the inorganic carbon produced by photo-oxidation of DOC after first removing inorganic carbon from the samples by acidification and gas flushing²⁸.

FIG. 2 Illustration of linkages among drought, acidification, DOC concentration and UV-B penetration depth in lakes. Solid-headed arrows link processes; open-headed arrows indicate changes in frequency, intensity or concentrations of constituents or processes. DOC concentration is affected by the mechanisms discussed in the text including acidification, Al increase and hydrological change (drought), but may also be affected by changes in DOC decomposition rate induced by changes in UV-B penetration depth. In Swan Lake, the principal mechanism for DOC decline in 1988 (Fig. 1e) was drought-induced acidification.



deposition zones are most at risk. This increased acidity will be accompanied by a decline in DOC concentrations, if the pH of the lake drops to ≤ 5.0 , as happened in Swan Lake. In consequence, the depth of underwater UV-B penetration will increase; this will compound the stress already on biota from the increased acidity.

The level of UV-B irradiation of hundreds of thousands²⁶ of eastern North American lakes has increased because of stratospheric ozone depletion^{1,27}. Further increases, especially in the shorter, more photochemically active UV-B wavelengths, are expected over the next six decades¹. Precipitation is also most acidic in the eastern region of North America²⁵. Despite recent declines in anthropogenic SO₂ emissions²⁶, deposition rates of sulphur in this region will exceed those in non-industrialized areas for the foreseeable future²⁶. Because global emissions of greenhouse gases will also continue to increase for some time, the detrimental interaction of global climate change, acid deposition and increased UV-B irradiance described here will persist in this region. □

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- Kerr, J. B. & McElroy, C. T. *Science* **262**, 1032–1034 (1993).
- Schindler, D. W., Jefferson Curtis, P., Parker, B. R. & Stainton, M. P. *Nature* **379**, 705–708 (1996).
- Keller, W. *Can. J. Fish. Aquat. Sci.* (Suppl. 1) **49**, 3–7 (1992).
- Dixit, S. S., Dixit, A. S. & Smol, J. P. *Can. J. Fish. Aquat. Sci.* **46**, 1309–1312 (1989).
- Dillon, P. J., Reid, R. A. & Girard, R. *Wat. Air Soil Pollut.* **31**, 59–65 (1986).
- Keller, W., Pitblado, J. R. & Carbone, J. *Can. J. Fish. Aquat. Sci.* (suppl. 1) **49**, 25–32 (1992).
- Vandermeulen, H., Jackson, M. B., Rodrigues, A. & Keller, W. *Crypt. Bot.* **3**, 123–132 (1993).
- MacIsaac, H. J., Keller, W., Hutchinson, T. C. & Yan, N. D. *Wat. Air Soil Pollut.* **31**, 791–797 (1986).
- Yan, N. D., Keller, W., MacIsaac, H. J. & McEachern, L. J. *Ecol. Applic.* **1**, 52–65 (1991).
- Keller, W. & Pitblado, J. R. *Wat. Air Soil Pollut.* **29**, 285–296 (1986).
- Dillon, P. J. & LaZerte, B. D. *Envir. Pollut.* **77**, 211–217 (1992).
- Bayley, S. E., Behr, R. S. & Kelley, C. A. *Wat. Air Soil Pollut.* **31**, 104–114 (1986).
- Bodo, B. & Dillon, P. J. in *Time Series Analysis in Hydrology and Environmental Engineering* (eds Hipel, K. W., McLeod, A. I., Panu, U. S. & Singh, V. P.) 285–298 (Kluwer, Boston, 1994).
- Kelso, J. R. M., Shaw, M. A. & Jeffries, D. S. *Envir. Pollut.* **78**, 65–71 (1992).
- Schindler, D. W. *et al. Proc. R. Soc. Edinb.* **97B**, 193–226 (1991).
- Dillon, P. J., Reid, R. A. & de Grosbois, E. *Nature* **329**, 45–48 (1987).
- Effler, S. W., Schafran, G. C. & Driscoll, C. T. *Can. J. Fish. Aquat. Sci.* **42**, 1707–1711 (1985).
- Schindler, D. W. *et al. Hydrobiol.* **229**, 1–22 (1992).
- Almer, B., Dickson, W., Ekström, C. & Hörnström, E. in *Sulphur Pollution in the Aquatic Ecosystem* (ed. Nriagu, J.) 271–311 (Wiley, New York, 1978).
- Spry, D. J. & Wiener, J. G. *Envir. Pollut.* **71**, 243–304 (1991).
- Helmer, E. H., Urban, N. R. & Eisenreich, S. J. *Biogeochemistry* **9**, 247–276 (1990).
- Devito, K. J., Dillon, P. J. & LaZerte, B. D. *Biogeochemistry* **8**, 185–204 (1989).
- Scully, N. M. & Lean, D. R. S. *Arch. Hydrobiol. Beih., Ergebn. Limnol.* **45**, 135–144 (1994).
- Manabe, S. & Wetherald, R. T. *Science* **232**, 626–628 (1986).
- Husar, R. B., Sullivan, T. J. & Charles, D. F. in *Acidic Deposition and Aquatic Ecosystems: Regional Case Studies* (ed. Charles, D. F.) 65–82 (Springer, New York, 1991).
- Jeffries, D. S., Lam, D. C. L., Wong, I. & Bixam, R. M. *Envir. Monitor. Assess.* **23**, 99–113 (1992).
- Stolarski, R. S., Bloomfield, P., McPeters, R. D. & Herman, J. R. *Geophys. Res. Lett.* **18**, 1015–1018 (1991).
- Handbook of analytical methods for environmental samples* (Lab. Services Branch Rep., Ontario Ministry of the Environment and Energy, Toronto, 1981).

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A latitudinal gradient in carbon turnover times in forest soils

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ATTEMPTS to model the global carbon cycle, and anthropogenic modifications to carbon flow between the atmospheric, oceanic and terrestrial carbon reservoirs, commonly rely on values assumed for the ¹³C/¹²C ratio and 'bomb-spike' ¹⁴C signature of carbon in each reservoir^{1,2}. A large proportion of the carbon in the terrestrial biosphere resides in the soil organic carbon (SOC) pool³, most of which is derived from plants that assimilate carbon via the C₃ photosynthetic pathway⁴. Here we report measurements of the ¹³C and ¹⁴C signatures of particulate organic carbon from surface soils of C₃ biomes from a global distribution of low-altitude, non-water-stressed locations. We find that there is currently a latitudinal gradient in the signature, with low-latitude soils being relatively depleted in ¹³C. The ¹⁴C signatures indicate that today's gradient is due to a latitudinal gradient in the residence time of the soil organic carbon, coupled with anthropogenic modifications to the ¹³C/¹²C ratio of atmospheric CO₂ (for example by fossil-fuel burning⁵). The long residence times (tens of years) of particulate organic carbon from high-latitude soils provide empirical evidence that if fluxes of carbon from vegetation to the soil increase, these soils have the capacity to act as a carbon sink on decadal timescales.

The mechanisms by which isotopic fractionation occurs during photosynthesis in C₃ plants are well known⁶. In addition, several studies have demonstrated how environmental factors (such as water availability⁷, irradiance⁸, altitude⁹ and respired-CO₂ reutilization¹⁰) can affect the $\delta^{13}\text{C}$ value of C₃ plant tissue, in some cases at the ecosystem level⁷. In contrast, there have been few attempts to examine how these factors affect the isotopic composition of organic carbon in the much larger SOC pool, or to place observational constraints on the isotopic composition of the SOC pool at a global level.

Balesdent *et al.*¹¹ have shown that there is no carbon-isotope fractionation during the early decay of fallen plant material. Therefore, the $\delta^{13}\text{C}$ value of surface soil also provides a useful proxy for the 'bulk' $\delta^{13}\text{C}$ value of the overlying vegetation where SOC turnover times are short, by smoothing out variations due to species-specific and tissue-specific fractionation effects which occur between individual plants, as well as reducing effects on

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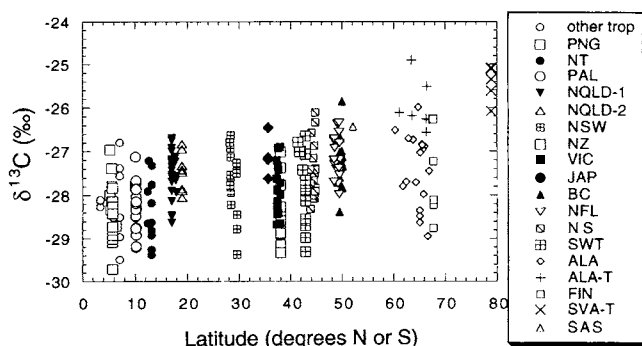


FIG. 1 Latitudinal gradient in the $\delta^{13}\text{C}$ value of surface soil samples analysed in this study. The difference between the means of high- and low-latitude populations is significant at the 99.9% confidence level using a 2-tailed *t*-test. Individual samples represent the 0–2 cm depth interval of soils collected at 3–5 locations over a distance of 10–20 m. Samples were wet-sieved and the $<500\ \mu\text{m}$ fraction treated overnight at room temperature with 1N HCl to remove carbonates. The $\delta^{13}\text{C}$ value of SOC was determined by closed-tube combustion, with an uncertainty of $\pm 0.1\text{‰}$ (all results reported as per mil deviations from the international PDB standard). Experimental techniques are described in detail in Bird *et al.*¹⁹. Sample localities (all forests unless otherwise noted), are as follows: SVA-T, Svalbard tundra (78°N); FIN, Finland (69°N); ALA-T, Alaskan tundra (60 – 67°N); ALA, Alaska (60 – 65°N); SWT, southwestern Tasmania (43°S); NS, Nova Scotia (44°N); NFL, Newfoundland (49°N); BC, British Columbia (50°N); SAS, Saskatchewan (53°N); JAP, Japan (36°N); VIC, Victoria (38°S); NZ, New Zealand (39°S); NSW, New South Wales (29°S); NQLD, north Queensland (17 – 19°S); PAL, Palawan (10°S); NT, Northern Territory (13°S); PNG, Papua New Guinea (5°S); other trop., other samples from Brazil, Cameroon and Mindanao (3 – 7°N and S).

fractionation in individual plants which result from fluctuating environmental conditions.

We determined the $\delta^{13}\text{C}$ value and ^{14}C activity of surface soils (0–2 cm depth, $<500\ \mu\text{m}$ fraction, carbonate-free) from globally distributed localities. At most localities, between 10 and 30 samples were collected over distances of kilometres to hundreds of kilometres. To minimize variability in isotopic composition due to the presence of C_4 plants, only soils from beneath closed forests, and soils from high-latitude grassland/tundra regions, were sampled. Also in order to minimize local variability, the samples were collected only at low-altitude sites ($<1,000\text{ m}$ above sea level) and from regions where the vegetation does not experience long periods of low water availability. Thus the samples represent 'end-members' for soils under C_3 vegetation. SOC from soils where C_4 vegetation is present, as well as soils from high altitudes or where vegetation is water-stressed, will have $\delta^{13}\text{C}$ values variably enriched in ^{13}C compared to the samples in this study.

Figure 1 shows the $\delta^{13}\text{C}$ value for all samples, plotted with respect to sample latitude. The standard deviation (1σ) of samples collected in a single locality averages $\pm 0.63\text{‰}$, similar to results obtained from multiple samples collected in a single stand of temperate forest by Balesdent *et al.*¹¹. There is an overall increase in SOC $\delta^{13}\text{C}$ value of $\sim 1\text{‰}$ from low-latitude to high-latitude forests, whereas high-latitude grassland/tundra samples (SVA-T, ALA-T in Fig. 1) are $\sim 1.5\text{‰}$ more enriched in ^{13}C than high-latitude forest samples. SOC from low-latitude soils (0 – 20°N or S) has average $\delta^{13}\text{C}$ values of $-28.3 \pm 0.6\text{‰}$ (1σ), mid-latitude soils (20 – 40°) have values of $-27.7 \pm 0.6\text{‰}$ and high-latitude soils (40 – 90°) have values of $-27.3 \pm 0.7\text{‰}$. The average for all forest soils is $-27.7 \pm 0.8\text{‰}$ (1σ ; $n = 320$), whereas the high-latitude grassland/tundra samples have an average $\delta^{13}\text{C}$ value of $-25.8 \pm 0.5\text{‰}$.

The observed gradient in $\delta^{13}\text{C}$ values from low to high latitudes could have several causes. It could represent (1) temperature effects on photosynthetic fractionation, (2) isotopic effects of forest type, or (3) differing rates of incorporation of anthropogenically produced atmospheric CO_2 into the soils. The $\delta^{13}\text{C}$ value

of atmospheric CO_2 has been decreasing since the industrial revolution, owing to the addition of low- ^{13}C carbon dioxide from biomass burning and fossil-fuel combustion⁵, from values around -6.5‰ to modern values of -7.8‰ . If the residence time of carbon in a surface soil is short, then the $\delta^{13}\text{C}$ value of SOC will decrease in parallel with the atmosphere, as plants assimilate the low- ^{13}C carbon dioxide and transfer it to the soil as organic matter. If the residence time of carbon in a surface soil is long, then SOC will retain a 'memory' of an older atmospheric $\delta^{13}\text{C}$ value, and the decrease in atmospheric CO_2 $\delta^{13}\text{C}$ value will manifest itself much more slowly in the SOC $\delta^{13}\text{C}$ value.

To distinguish between the above first two alternatives and the third, the ^{14}C activities of selected soils were determined in order to calculate the residence time of organic carbon in the soil. In order to reduce local variability, 10–20 individual samples from selected regions were combined to provide 'average' regional samples. Because there are several different components in SOC, which may have different residence times in the soil, the 63 – $500\ \mu\text{m}$ fraction was chosen for analysis. As degradation reduces particle size, this fraction can be expected to have a comparatively short residence time compared to the bulk soil, and hence anthropogenic effects on the $\delta^{13}\text{C}$ value will be more readily distinguishable in this fraction compared with organic carbon in the finer fractions.

Figure 2 shows the $\delta^{13}\text{C}$ value of the 63 – $500\ \mu\text{m}$ fraction of the combined 'average' regional soils analysed, plotted with respect to latitude. It also shows the $\delta^{13}\text{C}$ value of the soil corrected to a pre-industrial atmospheric CO_2 $\delta^{13}\text{C}$ value of -6.5‰ , based on a residence time for the carbon calculated from the ^{14}C activity of the sample (see Fig. 2 legend). The residence times for carbon in the 63 – $500\ \mu\text{m}$ fraction for samples in tropical and temperate latitudes are all less than five years, while residence times for carbon in high-latitude samples are tens of years. Longer residence times in high-latitude soils are due both to colder temperatures and shorter growing seasons.

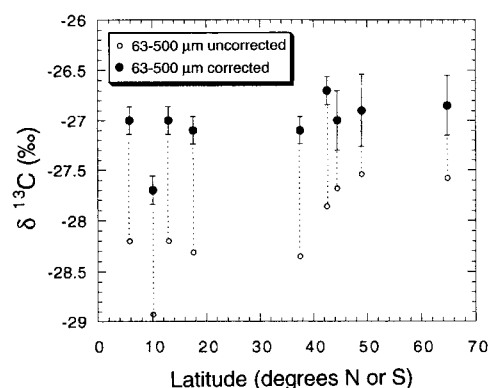
When the $\delta^{13}\text{C}$ results for the average soils are corrected for the effects of residence time (that is, the presence of older carbon), to a pre-industrial atmospheric CO_2 $\delta^{13}\text{C}$ value of -6.5‰ , all samples except one converge on a value of $-27.0 \pm 0.3\text{‰}$. It should be noted that this correction does not take into account the nonlinearity of the decrease in atmospheric CO_2 $\delta^{13}\text{C}$ value since industrialization. This could have a small effect on the corrected $\delta^{13}\text{C}$ values of those samples with long residence times, if the flux of carbon into or out of the soil has changed in the past few decades, but this effect is negligible compared with the error quoted above.

The sample representing the moist tropical forests of Palawan remains 0.5 – 1.0‰ depleted in $\delta^{13}\text{C}$ after the residence-time correction is applied. This may be a real difference, resulting from the large-scale re-utilization of low- ^{13}C respired CO_2 (which is a common phenomenon in dense, high-productivity, moist tropical forests¹⁰) or may indicate that there is a small direct effect of temperature on SOC $\delta^{13}\text{C}$ values.

Apart from the Palawan sample, there appears to have been little difference in the bulk $\delta^{13}\text{C}$ value of forest soils analysed in this study (and therefore bulk standing biomass) before industrialization. On the other hand, high-latitude grassland/tundra SOC (and hence biomass) must have been at least 1‰ higher in ^{13}C than forest SOC before industrialization. This could be due either to a biome-wide average difference in the degree of respired CO_2 re-utilization between forests and grasslands, or to a biome-wide average difference in the magnitude of isotopic fractionation during the photosynthetic assimilation of carbon by plants which make up grasslands compared to those which make up forests.

Other studies of the latitude dependence of plant-tissue $\delta^{13}\text{C}$ values have reached conflicting conclusions. Körner *et al.*⁹ found that leaf $\delta^{13}\text{C}$ values increased with increasing latitude, whereas Stuiver and Braziunas¹² concluded that tree-ring cellulose $\delta^{13}\text{C}$ values decreased with increasing latitude. The SOC $\delta^{13}\text{C}$ results

FIG. 2 Absence of a latitudinal gradient in the $\delta^{13}\text{C}$ value of 'average' regional soils corrected for the presence of 'old' SOC using the ^{14}C activity of the sample (filled circles). Also shown are the uncorrected $\delta^{13}\text{C}$ values for each sample (open circles). The 'average' soil samples represent 10–20 individual soil samples collected from the same region, bulked, size-fractionated by wet-sieving and decarbonated with HCl (see Fig. 1 legend). The correction to a pre-industrial atmospheric CO_2 $\delta^{13}\text{C}$ value of -6.5‰ is based on the ^{14}C 'age' for carbon in the sample, which is used to assign a $\delta^{13}\text{C}$ value for atmospheric CO_2 at the time at which the CO_2 was assimilated into the standing biomass, according to the following equation: $\delta^{13}\text{C}_{\text{PI}} = \delta^{13}\text{C}_{1993} - \{\Delta^{13}\text{C}_{(1993-\text{PI})} - \Delta^{13}\text{C}_{(1993-t)}\}$, where $\delta^{13}\text{C}_{\text{PI}}$ is the $\delta^{13}\text{C}$ value of pre-industrial SOC, $\delta^{13}\text{C}_{1993}$ is the $\delta^{13}\text{C}$ value of SOC, $\Delta^{13}\text{C}_{(1993-\text{PI})}$ is the difference between the $\delta^{13}\text{C}$ value of CO_2 in the modern (1993) and pre-industrial atmospheres ($=1.3\text{‰}$), and $\Delta^{13}\text{C}_{(1993-t)}$ is the difference between the $\delta^{13}\text{C}$ value of CO_2 in the modern atmosphere, and the atmosphere at year t (where t is the calculated residence time for carbon in the sample). The theoretical ^{14}C activity of a sample containing carbon with a simple residence time, for 1993, increases rapidly from the atmospheric ^{14}C value of 113‰M (measured as the ^{14}C activity of the sample corrected for fractionation, relative to 0.95×10^{14} C activity of oxalic acid I standard) for a 1-year residence time, peaking at 127‰M for a residence time of 16 years and then dropping more slowly to 111‰M for a residence time of 100 years. It is therefore not possible to uniquely determine whether a measured ^{14}C activity lies on the leading or trailing limb of the curve. It is assumed that values $<118\text{‰}$ (which include all tropical soils) lie on the leading edge of the curve, consistent with the results from other studies which indicate short residence times for carbon in tropical soils². Residence times for values of greater than 118‰ (which include all high-latitude soils) are calculated assuming that the result could lie on either limb of the curve, and consequently the error in the applied correction is larger. It should be noted that of the three highest-latitude samples, the Nova Scotian sample (NS) has a highest ^{14}C activity, but also has the highest mean annual temperature ($\sim 7.5^\circ\text{C}$), and longest growing season. The other samples from Alaska (ALA) and Newfoundland (NFL) both have lower mean annual



temperatures ($\sim 1^\circ\text{C}$ and $\sim 4.5^\circ\text{C}$ respectively) and lower ^{14}C activities. Given that a prime determinant of soil respiration and SOC residence time is temperature^{13,16} these results suggest that the ^{14}C activities of at least the ALA and NFL samples lie on the trailing edge of the curve, and are more likely to relate to residence times of 30–50 years than 5–10 years. A much larger sample population would be required to confirm this, and hence the errors quoted for the residence times include both possibilities. The ^{14}C activities and calculated residence times for the samples are as follows (sample number given in parentheses): ALA (ANU-9482) $123.3 \pm 0.8\text{‰M}$ (collected 1994), 20 ± 13 yr; NFL (ANU-9355) $118.1 \pm 0.9\text{‰M}$ (1993), 27 ± 22 yr; NS (ANU-9357) $126.9 \pm 1.0\text{‰M}$ (1993), 19 ± 9 yr; TAS (ANU-9480) $112.3 \pm 0.8\text{‰M}$ (1994), ≤ 5 yr; VIC (ANU-9481) $114.4 \pm 0.8\text{‰M}$, (1994), ≤ 5 yr; NQLD (ANU-9356) $110.2 \pm 1.6\text{‰M}$ (1993), ≤ 5 yr; NT (ANU-9511) $115.3 \pm 0.9\text{‰M}$ (1994), ≤ 5 yr; PAL (ANU-9354) $116.5 \pm 1.1\text{‰M}$ (1993), ≤ 5 yr; PNG (ANU-9479) $118.0 \pm 0.8\text{‰M}$ (1992), ≤ 5 yr.

presented above suggest that the results of both studies may be robust, and that the combined effect of several processes that produce the observed relationships with latitude in plant-tissue $\delta^{13}\text{C}$ values appear to result in no large net latitude effect for bulk plant tissue or SOC $\delta^{13}\text{C}$ value. Although these results are based on samples carefully selected to exclude other sources of variation, it should be stressed that the conclusion that there was a minimal latitude- (temperature-) induced gradient in SOC $\delta^{13}\text{C}$ value in pre-industrial times in applicable to all soils under C_3 vegetation.

Residence times for carbon in the 63–500 μm fraction of the surface soils range from <5 years for tropical and temperate soil samples to 10–50 years for the high-latitude soil samples (see Fig. 2 legend). This means that SOC levels in low-latitude soils will respond rapidly to changes in carbon delivery rates, whereas high-latitude SOC levels will respond much more slowly.

The $\delta^{13}\text{C}$ values for SOC in the 5–63 μm , 2–5 μm and $<2\text{ }\mu\text{m}$ fractions of all the soils analysed are similar to, or higher than, the corresponding 63–500 μm fractions of the soils (M.I.B., unpublished data), implying that carbon in the finer fractions is similar in age, or older than, carbon in the 63–500 μm fractions, consistent with results from other studies.² Therefore, the 10–50 year residence time calculated for high-latitude SOC in the 63–500 μm fraction represents a minimum value for the average time required to transfer additional particulate carbon through the SOC pool. This time is long compared to values commonly assumed for turnover times for 'detrital' carbon at high latitudes, which are generally assumed to be <10 years (see, for example, ref. 13), and in the case of the three highest-latitude samples are more likely to be 30–50 years than <20 years (see Fig. 2 legend).

Schimel *et al.*¹³ predicted that a latitudinal gradient in the $\delta^{13}\text{C}$ value of SOC should have developed in response to anthropogenic perturbations to atmospheric CO_2 $\delta^{13}\text{C}$ value, as a result of longer turnover times in high-latitude soils relative to low-latitude soils. The $\delta^{13}\text{C}$ and ^{14}C results presented above confirm this prediction, and thus support the results of studies such as that of Ciais *et al.*¹ who used a predicted latitudinal $\delta^{13}\text{C}$ gradient to conclude that northern temperate and boreal ecosystems acted as a net sink for

3.5 Gt of carbon in 1992, although increased uptake during 1992 may have been related to a major climate anomaly at that time¹⁴.

General mechanisms that have been proposed to account for the existence of a high-latitude terrestrial sink include the re-growth of high-latitude forests across previously denuded regions, and/or an increase in the uptake of carbon by existing forests due to anthropogenic CO_2 release and nitrogen fertilization^{15,16}.

Regardless of the mechanism, any increase in carbon uptake by standing biomass will result in a disequilibrium increase in soil carbon storage at high latitude (and hence a sink for atmospheric CO_2), owing to the decadal timescales involved in reaching equilibrium with newly elevated rates of carbon delivery to the soil. Although the ^{14}C -based turnover times presented in this study refer only to the 63–500 μm fraction of particulate surface SOC, they nonetheless represent minimum values for all particulate SOC in the samples, as discussed above. Soils at low latitude will also respond to elevated rates of carbon delivery, but owing to the fast turnover times, they have only a limited capacity to affect atmospheric CO_2 levels.

Our results provide support for the proposition that high-latitude forest soils have the capacity to act as a net sink for anthropogenic CO_2 . It should be noted that high-latitude soil respiration rates are particularly sensitive to temperature changes^{17,18}, so it is possible that increases in carbon storage may be at least partly offset in the future by any increase in temperature. □

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1. Ciais, P. *et al.* *J. geophys. Res.* **100**, 5051–5070 (1995).
2. Trumbore, S. E. *Global biogeochem. Cycles* **7**, 275–290 (1993).
3. Siegenthaler, U. & Sarmiento, J. L. *Nature* **365**, 119–125 (1993).
4. Lloyd, J. J. & Farquhar, G. D. *Oecologia* **99**, 201–215 (1994).
5. Freidli, H. *et al.* *Nature* **324**, 237–238 (1986).
6. Farquhar, G. D., von Caemmerer, S. & Berry, J. A. *Planta* **149**, 78–90 (1980).
7. Stewart, G. R., Turnbull, M. H., Schmidt, S. & Erskine, P. D. *Aust. J. Pl. Physiol.* **22**, 51–55 (1995).
8. Ehrlinger, J. R., Field, C. B., Lin, Z.-F. & Kuo, C.-Y. *Oecologia* **70**, 520–526 (1986).
9. Körner, Ch., Farquhar, G. D. & Roksandic, Z. *Oecologia* **74**, 623–632 (1988).
10. Van der Merwe, N. J. & Medina, E. *Geochim. cosmochim. Acta* **53**, 1091–1094 (1989).
11. Balesdent, J., Girardin, C. & Manotti, A. *Ecology* **74**, 1713–1721 (1993).
12. Stuiver, M. & Braziunas, T. F. *Nature* **328**, 58–60 (1987).

13. Schimel, D. S. *et al. Global biogeochem. Cycles* **8**, 279–293 (1994).
14. Schimel, D. S. *Global Change Biol.* **1**, 77–91 (1995).
15. Kauppi, P. E., Mielikäinen, K. & Kuusela, K. *Science* **256**, 70–74 (1992).
16. Schindler, D. W. & Bayley, S. E. *Global biogeochem. Cycles* **7**, 717–733 (1993).
17. Lloyd, J. & Taylor, J. A. *Functional Ecol.* **8**, 315–323 (1994).
18. Oechel, W. C. *et al. Nature* **361**, 520–523 (1993).
19. Bird, M. I., Haberle, S. G. & Chivas, A. R. *Global biogeochem. Cycles* **8**, 13–22 (1994).

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Effects of mass extinctions on biodiversity

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QUANTITATIVE studies of patterns of diversification and extinction throughout geological time have come to rely on databases assembled by Sepkoski¹. A new database has been presented by Benton², who argues that the increase in diversity is exponential, that “there is no need to assume equilibrium levels of global diversity, nor to apply logistic models to the investigation of past diversification patterns”, and that mass extinctions played no special role in the diversification of life. However, we find that diversification can be predicted by a simple model in which long periods of growth obeying a logistic equation are sporadically interrupted by brief events leading to mass extinction. The initial value of diversity immediately after a mass extinction, initial growth rate and subsequent equilibrium level determine each segment of the growth curve. The larger mass extinctions that came after long periods without one seem to have been followed by larger equilibrium levels of diversity.

We re-display Benton's data (see note 32 of ref. 2) in Fig. 1, together with the least-squares fit of an exponential curve to the data (marine families). The root mean square (r.m.s.) deviation is of the order of 130 families. Departures of the data curve from the exponential fit are highly correlated. The data and model intersect near the boundaries of the main geological eras (which by definition correspond to sudden drops in diversity). However, the fast rise of diversity in the Ordovician period followed by plateau-like behaviour in the rest of the Palaeozoic era is reminiscent of a logistic pattern³. It is interrupted by the great mass extinction at the end of the Permian period. Diversity also increased after the mass extinctions at the end of the Triassic and Cretaceous periods, although it began to level off after the Eocene epoch. Following Sepkoski^{3–5}, we propose that diversification is determined not by exponential but by logistic behaviour most of the time, and that this is interrupted by rare, brief (less than 1 million years (1 Myr)) catastrophic events leading to mass extinctions. This model requires *a priori* knowledge of the number and dates of

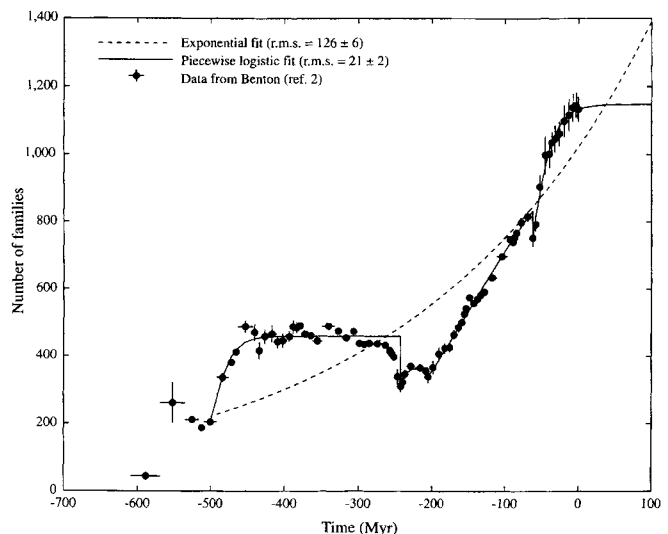


FIG. 1 Diversification of families of marine organisms through time: data and models. The circles are data from Benton², the broken line is an exponential fit to the data, also from Benton, and the solid line is a piecewise logistic fit to equation (1) (this work). Segments of logistic curves are interrupted by three mass extinctions in the Late Permian, Late Triassic and Late Cretaceous. The respective r.m.s. misfits are 126 ± 6 for the exponential fit and 21 ± 2 families for the logistic fit. Note that the two models predict quickly diverging values in the future. If we remove the first three (Vendian) data points, we have 75 data points. There are 73 degrees of freedom for the exponential fit ($75 - 2$ free parameters) and 57 for the multilogistic fit ($75 - 12$ (4 time segments with 3 free parameters each) $- 6$ (3 time constraints where the jump is specified)). The *F* (Snedecor) ratio of variances of residuals with respect to the two fits is 39, and the critical value $F_{0.995}(57; 73)$ at the 99.5% confidence level is on the order of 2. Hence the increase in quality of fit is significant to far better than that confidence level.

TABLE 1 Logistic model parameters

Data fitted	N_0	N_f	α	r.m.s.
1a. Vendian–Permian				
Maximum	47	475	0.67×10^{-4}	50
Mean	44	467	0.68×10^{-4}	40
Minimum	41	458	0.68×10^{-4}	33
1b. Ordovician–Permian				
Maximum	214	469	1.57×10^{-4}	23
Mean	204	459	1.57×10^{-4}	21
Minimum	195	449	1.56×10^{-4}	21
2a. Triassic–Cretaceous				
Maximum	327	$>10^9$	3.5×10^{-10}	17
Mean	310	21,723	2.7×10^{-7}	19
Minimum	294	3,869	1.8×10^{-6}	22
2b. Triassic*				
Maximum	327	373	5.8×10^{-4}	8
Mean	310	364	5.6×10^{-4}	7
Minimum	294	356	5.5×10^{-4}	6
3. Jurassic–Cretaceous				
Maximum	359	1,289	0.09×10^{-4}	13
Mean	340	1,120	0.12×10^{-4}	13
Minimum	320	1,021	0.15×10^{-4}	14
4. Cenozoic				
Maximum	778	1,183	0.55×10^{-4}	20
Mean	752	1,148	0.53×10^{-4}	15
Minimum	727	1,114	0.51×10^{-4}	12

The parameters were derived by nonlinear least-squares inversion of the data segments shown in Fig. 2.

* The fit of the Triassic data is ill constrained.

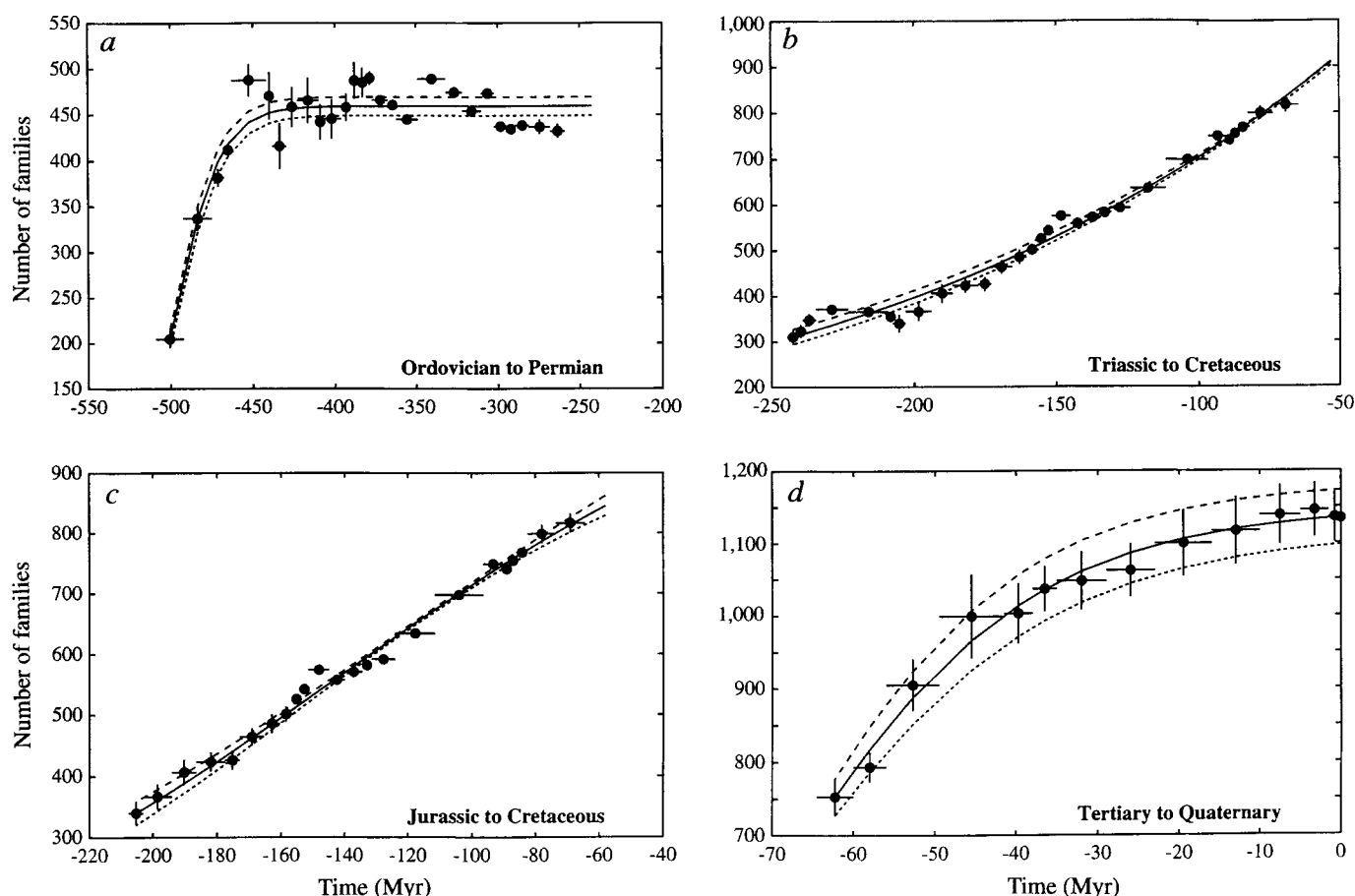


FIG. 2 A logistic fit from equation (1) to the family data (maximum, mean and minimum) of Fig. 1. *a*, Data from the Ordovician to the Permian. Maximum (dashed curve), mean (solid curve) and minimum (dotted curve) values are from Benton². All parameters are given in Table 1. Sepkoski³ considers that two intervals of logistic differentiation actually occurred within the Palaeozoic. However, we have not included the mass extinctions from the end of the Ordovician and the Late Devonian (see Fig. 8 in ref. 5): because the same plateau (asymptote) has been reached after both, and the total numbers are not yet very large, they do not appear to have had a large influence on the diversification pattern. Also, Benton's data² contain

fewer sampling intervals over the Cambrian than do Sepkoski's. In any case, sources of noise and uncertainty are such (for instance, particularly in the Vendian, that of 'Lagerstätten' or exceptional yet single fossil sites) that more elaborate treatment and curve fitting is not warranted. *b*, As *a*, but using data from the Triassic to the end of the Cretaceous; the fit, which ignores the mass extinction at the end of the Triassic, is acceptable but leads to unreasonable asymptotic values. *c*, As *b*, but for data from the Jurassic and Cretaceous only. The fit to the Triassic data, which is ill constrained because of the short time interval, is not shown. *d*, As above but for the Cenozoic data.

these events. The extinctions at the end of the Permian, Triassic and Cretaceous are the most prominent^{1,2}. For the Palaeozoic, one should add those at the end of the Ordovician and in the Late Devonian, with a relative magnitude that places them in the 'big five' events of the entire Phanerozoic eon⁶. For simplicity, we attempt to model only three of the five largest mass extinctions at the end of the Permian, Triassic and Cretaceous. We use marine families only, because they correspond to a more stationary, closed environment than terrestrial families.

The starting logistic differential equation is

$$dN/dt = \alpha N(N_t - N) \quad (1)$$

where N is the instantaneous number of families, N_t the equilibrium level, and α a second free parameter that governs the initial growth rate and the time taken to reach equilibrium values. Both free parameters can be determined from the data by nonlinear least-squares fitting. With initial condition $N(t=0) = N_0$, the solution³⁻⁵ is

$$N(t) = N_0 N_t [N_0 + (N_t - N_0) \exp(-\alpha N_t t)]^{-1} \quad (2)$$

The maximum growth rate occurs when $N = N_t/2$ and $(dN/dt)_m = \alpha N_t^2/4$, which occurs at time $t_m = (1/\alpha N_t) \ln(N_t/N_0 - 1)$.

We fitted equation (1) to the data from early Ordovician to the end of the Palaeozoic (Fig. 2*a*, where fits to the minimum and

maximum estimates of Benton are also shown). The fit is excellent, with a r.m.s. deviation of about 20 families. The asymptote is on the order of 460 families. Starting in the Vendian, rather than early Ordovician, makes little difference (Table 1). After the mass extinction at the end of the Permian, the number of families is drastically reduced. If we fit all of the data from the entire Mesozoic era to a single logistic curve, we find a reasonable fit (Fig. 2*b*), but meaningless α and N_t values (Table 1), owing to the omission of the extinction of the end of the Triassic. When the data to be fitted are restricted to the Jurassic and Cretaceous periods (Fig. 2*c*), there is a good fit (r.m.s. 13 families), with a more acceptable N_t of 1,120 families. We next fit the Cenozoic data after the end-Cretaceous mass extinction at the end of the Cretaceous, and found an excellent fit (Fig. 2*d*; r.m.s. of about 15 families), with a well-constrained N_t again on the order of 1,150.

The fit of the composite model resulting from four logistic curves separated by the three major mass extinctions is shown in Fig. 1. The total r.m.s. of residuals is on the order of 20 families, which is six times less than with the exponential fit. This is a highly significant improvement, verified using a Snedecor test (see Fig. 1 legend). Note that the Triassic segment is too short to be well constrained.

In slight contrast to our model, Sepkoski⁵ identified three separate evolutionary faunas (Cambrian, Palaeozoic and Modern), each going through initial diversification, dominance and decline. According to Sepkoski, each successive fauna dis-

plays a slower rate of diversification but a higher level of maximum diversity than the preceding one. Each fauna obeys a logistic equation that is nonlinearly coupled to the others. As a result, the quasi-equilibrium behaviour observed in the Palaeozoic would have resulted from two successive compensations: expansion of the Palaeozoic fauna first compensating the decline of the Cambrian fauna, and then expansion of the Modern fauna compensating decline in the Palaeozoic fauna. Mass extinctions in this model only punctuate the expansion or decline periods of individual evolutionary faunas. We believe that total diversity may be a more robust indicator, that plateau structures indicate asymptotic logistic behaviour and not simply successive compensations, and that mass extinctions have a fundamental influence in determining subsequent evolution (both initial diversification rate and equilibrium diversity following the extinction).

Parameter α (Table 1) is a measure of the time taken to rise from low to high values of N . More precisely, the time δt taken to go from 50% to 90% of the asymptotic value N_i is $(2.2/\alpha N_i)$, that is, respectively of the order of 50, 180 and 40 Myr for the Palaeozoic, Jurassic plus Cretaceous, and Cenozoic. Although uncertainties are quite large, there may be a relation between α and the severity of the preceding crisis. The more severe extinction at the end of the Permian leads to a smaller α than that of the end of the Cretaceous, and the values of α are roughly inversely proportional to the relative number of families having disappeared in the previous extinction.

The reason for the apparent growth of equilibrium levels (N_i) after several mass extinctions remains an open question. It may be that, after mass extinctions, diversification is accelerated owing to greater capacities of pioneering species, perhaps also influenced

by the added space made available to them. Immediately after a mass extinction, the number of families falls back to a value it had a long time before, when species were rather different. The growth of N_i between the Palaeozoic and Mesozoic may be due to the number of families after the extinction at the end of the Permian, the largest of all, returning to a value that it had exceeded for the previous 250 Myr. In contrast, after the mass extinction at the end of the Cretaceous, the number of families returned to the value it had only 30 Myr before. This may be one of the reasons why the equilibrium levels are similar for the Mesozoic and Cenozoic, or within the Palaeozoic.

Our study confirms basic ideas put forward by Sepkoski³⁻⁷, which were disputed by Hoffman⁸ and not mentioned by Benton². The model could be made more complex and the fit improved by introducing other extinctions, but this is not yet justified by the data. □

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1. Sepkoski, J. J. *Milwaukee Public Mus. Contrib. Biol. Geol.* **51**, 1–125 (1982).
2. Benton, M. J. *Science* **268**, 52–58 (1995).
3. Sepkoski, J. J. *Paleobiology* **4**(3), 223–251 (1978).
4. Sepkoski, J. J. *Paleobiology* **5**(3), 222–251 (1979).
5. Sepkoski, J. J. *Paleobiology* **10**(2), 246–267 (1984).
6. Sepkoski, J. J. in *Systematics, Ecology, and the Biodiversity Crisis* (ed. Eldredge, N.) 77–100 (Columbia Univ. Press, NY, 1992).
7. Sepkoski, J. J. in *The Unity of Evolutionary Biology* (ed. Dudley, E. C.) 210–236 (Dioscorides Press, Portland, OR, 1991).
8. Hoffman, A. *Arguments on Evolution—A Paleontological Perspective* 218–224 (Oxford Univ. Press, New York, 1989).

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Strategy for rapid immobilization of prey by a fish-hunting marine snail

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SOME venomous animals capture prey with remarkable efficiency and speed. The purple cone, *Conus purpurascens*, uses two parallel physiological mechanisms requiring multiple neurotoxins to immobilize fish rapidly: neuromuscular block¹, and excitotoxic shock. The latter requires the newly characterized peptide κ -conotoxin PVIa, which inhibits the *Shaker* potassium channel²⁻⁴, and δ -conotoxin PVIa⁵, which delays sodium-channel inactivation. Despite the extreme biochemical diversity in venoms, the number of effective strategic alternatives for prey capture are limited. How securely prey is initially tethered may strongly influence the venom strategy evolved by a predator.

We investigated the mechanisms underlying the rapid immobilization of prey by the fish-hunting snail *C. purpurascens*, which injects venom and tethers prey using a hollow harpoon-like tooth¹ (Fig. 1). A good strike results in almost instantaneous rigid paralysis accompanied by major fin tetanus (Fig. 1c). The few fish that recover are invariably paralysed later in a flaccid state, but usually the immobilized fish is immediately engulfed (Fig. 1d).

Thus two distinct paralytic strategies appear to be elicited by venom. In addition to neuromuscular transmission block causing flaccid paralysis, a sudden tetanus of prey (STOP) syndrome is also observed.

C. purpurascens venom contains at least three paralytic toxins, which act by blocking neuromuscular transmission; two of these, α A-conotoxin PIVa⁶, which targets the nicotinic acetylcholine receptor, and μ -conotoxin PIIa, which blocks skeletal muscle sodium channels, are not responsible for the rigid immobilization characteristic of STOP.

Individual venom components do not elicit the full STOP syndrome observed *in vivo*. However, injecting pairs of venom components fractionated by high-performance liquid chromatography (HPLC) together revealed two fractions which in combination elicited STOP: δ -conotoxin PVIa⁵, and a fraction that caused a 'fin-popping' activity (see Fig. 2). Injection of the 'fin-popping' fraction initially led to hyperactivity of the fish, followed by continuous contraction and extension of major fins, without immobilization or death. In contrast, δ -conotoxin PVIa caused quiescence and jaw extension (the 'lock-jaw symptom'), followed by extremely jerky swimming and a generalized rigid paralysis from which fish would not generally recover. The *C. purpurascens* STOP syndrome is a single, lethal 'fin-pop' in envenomed fish, and requires both components. Additional venom components probably contribute because onset of STOP with the two purified components is longer than the 1–2 seconds observed *in vivo*.

The 'fin-popping' fraction was purified to homogeneity from *C. purpurascens* venom; amino-acid sequence determination revealed a peptide of 27 amino acids (Fig. 2). Chemical synthesis of biologically active peptide and molecular cloning have been achieved. All data support the sequence CRIONQKCFQHLDDCCSRKCNRFNKCVC, where O represents 4-trans-hydroxyproline.

The ω - and δ -conotoxins, which target voltage-gated calcium and sodium channels, respectively, have the same arrangement of Cys residues¹. However, the 'fin-popping' peptide does not compete for binding with radiolabelled ω - or δ -conotoxins, suggesting it is neither an ω - nor a δ -conotoxin (data not shown). Evidence for

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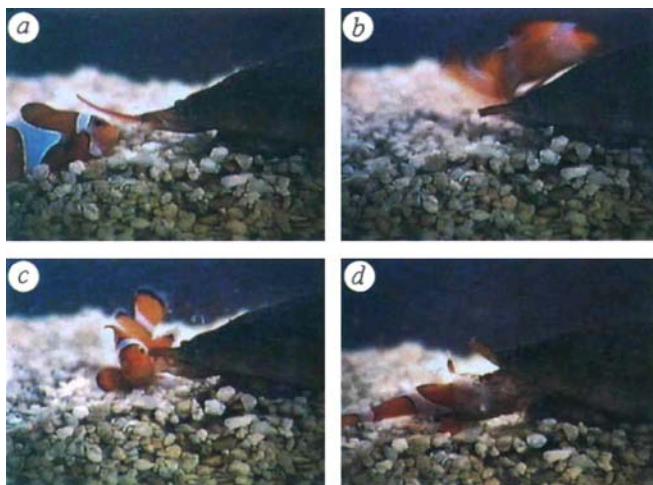


FIG. 1 *Conus purpurascens* rapidly capturing a clown fish. *a*, It extends its proboscis (the reddish structure) in preparation for stinging the clown fish, which was just introduced into the aquarium. *b*, *C. purpurascens* has just stung the fish, which immediately reacted and is swimming to the right of the snail; however, it is tethered to the snail's proboscis by a barbed harpoon-like tooth, which is hollow and allows venom to be injected into the fish. *c*, Within 2 s tetanus of the fins is observed; note the fully extended pectoral, dorsal and caudal fins. This is the STOP syndrome. The fish is immobilized, and is drawn by retraction of the proboscis towards the mouth (rostrum) of the cone shell. *d*, The tetanically immobilized fish is drawn within the rostrum of the snail (note that pectoral fins are still stiff) and completely engulfed. Digestion then proceeds and, in $\sim 1-2$ h, the snail regurgitates the scales and bones of the fish along with the single disposable harpoon tooth used to inject venom.

the mechanism underlying the 'fin-popping' activity was obtained with oocytes expressing the cloned *Shaker* K^+ channel²⁻⁴, as the toxin blocked the K^+ conductance of the expressed channel (Fig. 3a). A dose-response curve is shown in Fig. 3b, with half-maximal inhibitory concentrations (IC_{50}) ranging from 60 ± 3 nM (at 0 mV) to 70 ± 6 nM (at +60 mV), indicating only slight voltage dependence (Fig. 3c). Not surprisingly, given the very rapid physiological response *in vivo*, kinetics of block appear to be very fast. For the concentrations tested (20, 100 and 1,000 nM), steady-state block was reached in a few seconds (Fig. 3d). The 'fin-popping' peptide is the first member of a putative κ -conotoxin family of K^+ channel-targeted peptides; we call it κ -conotoxin PVIIA (κ -PVIIA). At 1 μ M it had no effect on type II Na^+ channels or the K^+ channels Kv1.1 or Kv1.4 from rat brain (data not shown).

Another component involved in STOP is the 29-amino-acid δ -conotoxin PVIA (δ -PVIA), which increases excitability in frog neuromuscular preparations⁵. Homology to δ -conotoxins from snail-hunting *Conus* spp. suggested that it might affect voltage-gated Na^+ channel function. We demonstrate that the normally rapid inactivation of both rat-brain type II Na^+ channels and rat hippocampal Na^+ currents is suppressed by δ -PVIA, thereby prolonging macroscopic Na^+ current duration (Fig. 4a-c). How-

ever, δ -PVIA had no effect when applied to the cytoplasmic side of inside-out oocyte patches expressing type II Na^+ channels. In addition, δ -PVIA did not inhibit any K^+ channel subtype tested (Kv1.1, Kv1.4 and *Shaker* H4).

Thus, when *C. purpurascens* stings fish, STOP is elicited by two excitotoxins, one increasing Na^+ influx, the other decreasing K^+ efflux, leading to powerful depolarization of cells. The acetylcholine receptor and voltage-gated Na^+ -channel inhibitors cause neuromuscular block and flaccid paralysis, which occur slowly owing to the dispersal time to synapses. However, κ -PVIIA and δ -PVIA cause rapid excitotoxic shock to immobilize prey quickly. When *C. purpurascens* harpoons fish they are not tethered securely, so rapid immobilization is required. Another cone shell, *C. geographus*, only uses neuromuscular block¹⁻⁷; because it captures prey securely using a net strategy, only stinging when the prey cannot escape, there is no need to use excitotoxins to cause rapid immobilization.

In general, evolution of excitotoxins may be influenced by how well prey is initially tethered. A sea snake that has securely impaled a small fish with venom fangs only uses neuromuscular block, whereas sea anemones, which initially contact their prey with a single tentacle, have convergently evolved Na^+ and K^+

FIG. 2 *C. purpurascens* specimens were milked for venom as previously described⁶. The venom was pooled, and fractionated by HPLC. *a*, The elution profile. The peaks indicated by the bar (right) contain the lockjaw peptide (δ -conotoxin PVIA), the purification and biochemical characterization of which has been described⁵. The peak indicated by the arrow is the fin-popping peptide (κ -conotoxin PVIIA). The peak was rechromatographed twice by HPLC with the standard TFA/acetonitrile buffer system; the gradient was 12–30% acetonitrile, 0.1% TFA (*b*, *c*). The fin-popping peptide elutes at 22% acetonitrile. An amino-acid sequence determination of the fin-popping peptide gave an unambiguous sequence assignment indicating a new 27-amino-acid peptide with 6 Cys residues (see text). In *d*, an equal amount of native and synthetic peptide were mixed and coeluted at 22% acetonitrile as a single homogeneous peak, indicating that the sequence assignment could be verified by synthesis.

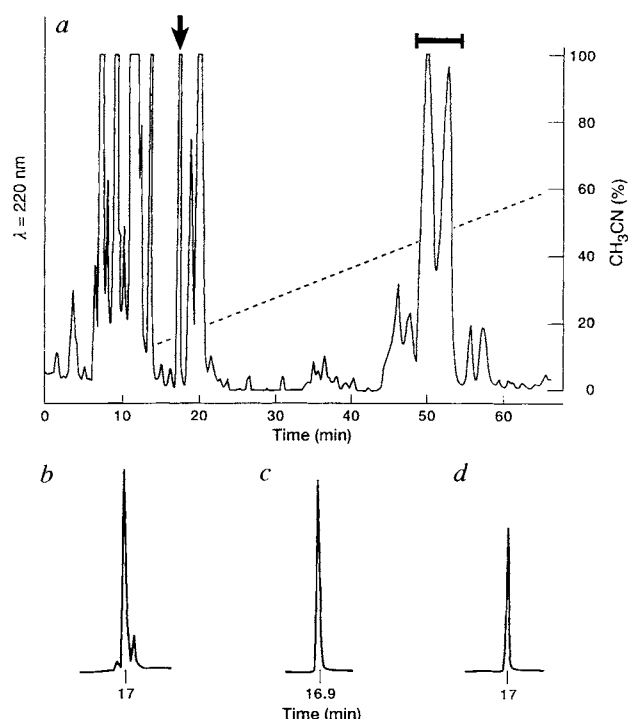


FIG. 3 κ -Conotoxin PVIIA blocks the *Shaker* K⁺ channel. *a*, Whole-cell currents from an oocyte expressing *Shaker* H4 K⁺ channels¹⁵ evoked by test potentials of -80 to $+60$ mV in steps of 10 mV (top). Presence of 200 nM κ -conotoxin PVIIA in the bath solution reduces the peak current amplitude to about 20% of the control value (middle) in a reversible manner (bottom). *b*, Dose-response curve for the block by κ -conotoxin PVIIA at a test potential of $+40$ mV. *c*, IC₅₀ of the block by κ -conotoxin PVIIA at different test potentials (mean $\pm$ s.e.m., $n = 5$). *d*, Example of the kinetics of the block by 100 nM κ -conotoxin PVIIA. Steady state of block was reached in less than 5 s and the wash-out was complete in less than 15 s ($n = 8$). Identical kinetic results were obtained with 20 nM ($n = 5$) and 1 μ M κ -conotoxin PVIIA ($n = 6$) indicating a very fast block of the toxin.

METHODS. Capped run-off *Shaker* cRNA for injection into *Xenopus* oocytes was synthesized using a template with a T7 promoter by a standard protocol¹⁶. Oocyte injection was performed as described¹⁷. Kv1.1 (ref. 18) and Kv1.4 (ref. 19) were recloned by the polymerase chain reaction (PCR). Whole-cell currents were measured with a two-electrode voltage clamp¹⁷. Current records were low-pass filtered at 1 kHz (-3 -dB) and sampled at 4 kHz. The bath solution was normal frog Ringer's (NFR) containing (in mM): 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 10 HEPES, pH 7.2 (NaOH). To estimate the IC₅₀ of κ -conotoxin PVIIA, whole-cell currents of oocytes expressing *Shaker* H4 K⁺ channels were measured while the toxin concentration in the bath was successively increased. The peak current at a given voltage was measured and plotted against the toxin concentration. Dose-response curves were fitted to the equation $y = [1 + (T/IC_{50})^n]^{-1}$, where T is the toxin concentration and n the Hill coefficient, which was about 1 . To estimate the kinetics of block, whole-cell currents were measured using a small bath chamber (volume < 100 μ l) with a perfusion of more than 10 ml min⁻¹. Cells were depolarized to a test potential of $+40$ mV from a holding potential of -100 mV with a stimulus frequency of 2 Hz. Higher-stimulus frequencies are inappropriate owing to the inactivation of the *Shaker* channels. The peak current amplitude was plotted against time. For toxin application the perfusion was stopped and 1 ml of the toxin solution was added directly to the bath solution. All electrophysiological experiments were performed at room temperature (19 – 22 °C).

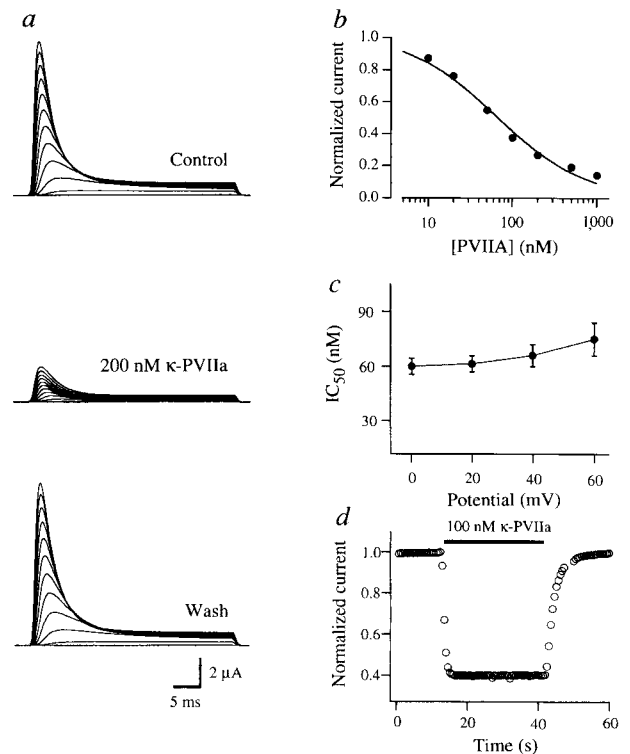
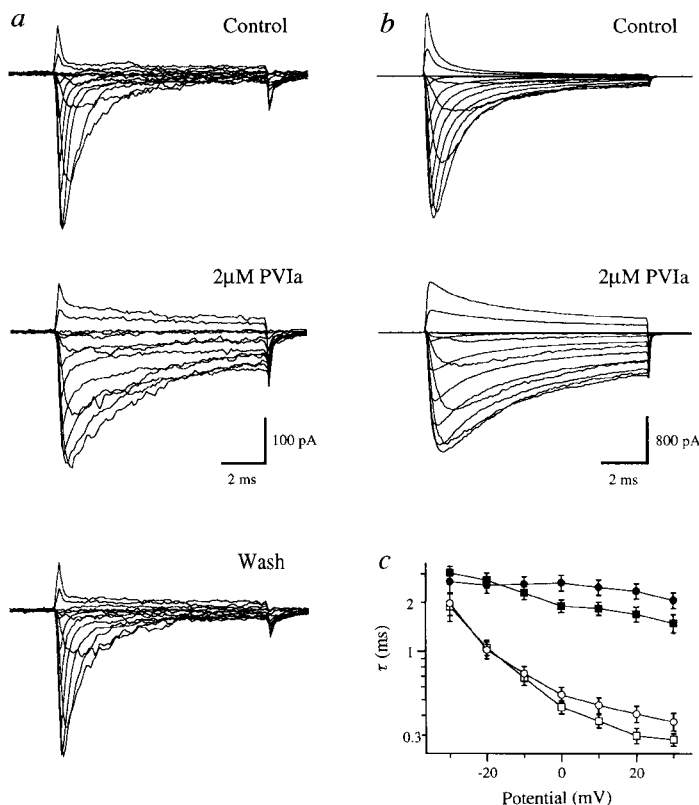


FIG. 4 δ -Conotoxin PVIA slows the fast inactivation of sodium currents. *a*, Sodium currents recorded from a nucleated patch from a hippocampal cell in culture. Currents were evoked by test potentials of -60 to $+60$ mV in steps of 10 mV from a holding potential of -80 mV. Top, control; middle, in the presence of 2 μ M PVIA; bottom, wash. *b*, Sodium currents from an outside-out macropatch excised from a *Xenopus* oocyte expressing rat-brain Na channel II. Patches were depolarized from a holding potential of -100 mV to test potentials ranging from -80 to $+60$ mV in steps of 10 mV. Top, control; bottom, in the presence of 2 μ M PVIA. *c*, Time constants of inactivation, τ , of the sodium currents plotted as a function of the test potential for nucleated patches from hippocampal neurons (squares, $n = 11$) and outside-out macropatch excised from an oocyte expressing rat-brain sodium channel II (circles, $n = 10$). Open symbols represent the values under control conditions, closed symbols after addition of 2 μ M PVIA. Values are mean $\pm$ s.e.m., from nonlinear least-square fits according to sodium current kinetics²⁰ assuming one inactivation gate.

METHODS. Rat hippocampal cultures were obtained²¹ and recordings were taken after 4 – 7 days in culture. The processes of the neurons precluded a good voltage control during clamp experiments in the whole-cell configuration²² so nucleated patch recordings²³ were performed. The pipettes had resistances of 2 – 3 M Ω and contained (in mM): 100 CsCl, 10 NaCl, 20 phosphocreatine, 50 units of creatine phosphokinase per ml, 4 MgATP, 10 EGTA and 10 HEPES-CsOH, pH 7.2 . The bath solution contained (in mM): 140 NaCl, 2.8 KCl, 2 CaCl₂, 2 MgCl₂ and 10 HEPES-NaOH, pH 7.2 . Currents were measured with an EPC-9 patch clamp amplifier driven by the Pulse+PulseFit software package (HEKA Elektronik, Lambrecht, Germany). Current records were low-pass filtered at 3 kHz (-3 -dB) and sampled at a rate of 10 kHz. Leak and capacitive currents were corrected on-line with a P/n method. Rat-brain Na⁺ channel II cRNA²⁴ for injection into *Xenopus* oocytes was synthesized using a template with an SP6 promoter. Currents were recorded 2 – 5 days after injection. Recording pipettes with resistances of 0.8 – 1.4 M Ω were either filled with (in mM) 100 KCl, 16 NaCl, 1.8 EGTA and HEPES-KOH, pH 7.2 , for outside-out patches, or with NFR for inside-out patches. Current records were low-pass filtered at 5 kHz (-3 -dB) and sampled at 20 kHz. The bath solution contained NFR for outside-out recordings and (in mM) 100 KCl, 16 NaCl, 1.8 EGTA, HEPES-KOH, pH 7.2 , for inside-out recordings. As PVIA is very hydrophobic, a concentrated stock solution of the toxin was added to the bath chamber (0.8 – 1.2 ml) with a Gilson pipetter. The indicated toxin concentration correspond to the final concentration in the bath chamber.



channel-targeted excitotoxins⁸⁻¹⁴ analogous to those used by *C. purpurascens*. □

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1. Olivera, B. M. et al. *Science* **230**, 1338–1343 (1985).
2. Kamb, A., Iverson, L. E. & Tanouye, M. A. *Cell* **50**, 405–413 (1987).
3. Papazian, D. M., Schwarz, T. L., Tempel, B. L., Jan, Y. N. & Jan, L. Y. *Science* **237**, 749–753 (1987).
4. Pongs, O. et al. *EMBO J.* **7**, 7087–7096 (1988).
5. Shon, K. et al. *Biochemistry* **34**, 4913–4918 (1995).
6. Hopkins, C. et al. *J. Biol. Chem.* **270**, 22361–22367 (1995).
7. Olivera, B. M. et al. *Science* **249**, 257–263 (1990).
8. Beress, L., Wunderer, G. & Wachter, E. *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 985–988 (1977).
9. Rathmayer, W. *Adv. Cytopharmacol.* **3**, 335–344 (1979).
10. Lazdunski, M. et al. *Ann. N. Y. Acad. Sci.* **479**, 204–220 (1987).
11. Karlsson, E. et al. *Toxicol.* **29**, 1168 (1991).
12. Aneiros, A. et al. *Biochim. biophys. Acta* **1157**, 86–92 (1993).
13. Castañeda, O. et al. *Toxicol.* **33**, 603–613 (1995).
14. Schweitz, H. et al. *J. Biol. Chem.* **270**, 25121–25126 (1995).
15. Kamb, A., Tseng-Crank, Z. & Tanouye, M. A. *Neuron* **1**, 421–430 (1988).
16. Krieg, P. A. & Melton, D. A. *Meth. Enzym.* **155**, 397–415 (1987).
17. Stühmer, W. *Meth. Enzym.* **207**, 319–339 (1992).
18. Baumann, A., Grupe, A., Ackermann, A. & Pongs, O. *EMBO J.* **7**, 2457–2463 (1988).
19. Stühmer, W. et al. *EMBO J.* **8**, 3235–3244 (1989).
20. Hodgkin, A. L. & Huxley, A. F. *J. Physiol., Lond.* **117**, 500–544 (1952).
21. Seifert, W. et al. in *Neurobiology of the Hippocampus* (ed. Seifert, W.) 109–135 (Academic, London, 1983).
22. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch.* **391**, 85–100 (1981).
23. Sather, W., Dieudonne, S., MacDonald, J. & Ascher, P. *J. Physiol., Lond.* **450**, 643–672 (1992).
24. Noda, M. et al. *Nature* **320**, 188–191 (1986).

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Left-right asymmetric expression of the TGF β -family member *lefty* in mouse embryos

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EXAMPLES of lateral asymmetry are often found in vertebrates, such as the heart being on the left side, but the molecular mechanism governing the establishment of this left-right (L-R) handedness is unknown¹. A diffusible morphogen may determine L-R polarity², but a likely molecule has not so far been identified. Here we report on the gene *lefty*, a member of the transforming growth factor- β family, which may encode a morphogen for L-R determination. Lefty protein contains the

cysteine-knot motif³ characteristic of this superfamily^{4,5} and is secreted as a processed form of relative molecular mass 25K–32K. Surprisingly, *lefty* is expressed in the left half of gastrulating mouse embryos. This asymmetric expression is very transient and occurs just before the first sign of lateral asymmetry appears. In the mouse mutants *iv* and *inv*, which cause *situs inversus*, the sites of *lefty* expression are inverted, indicating that *lefty* is downstream of *iv* and *inv*. These results suggest that *lefty* may be involved in setting up L–R asymmetry in the organ systems of mammals.

By applying a systematic subtraction procedure to the P19 embryonal carcinoma cells⁶, we have recently isolated a number of undifferentiated cell-specific complementary DNA clones⁷. One such clone (549) was a new gene related to transforming growth factor- β (TGF β) (Fig. 1), which we call *lefty*. The six cysteine residues that are conserved among TGF β -related proteins and which are necessary to form the cysteine-knot structure³, are also found in Lefty at the predicted positions (Fig. 1c). However, the entire sequence of Lefty shares only 20–25% similarity with known members of the TGF β superfamily.

The *lefty* gene product has unique structural features. First, whereas the carboxy terminus of the TGF β -related proteins is usually CX₁CX₁, Lefty has a longer C-terminal sequence, CX₁CX₁₃. Second, Lefty lacks the cysteine residue required for dimer formation through the disulphide linkage (Fig. 1b, c). Finally, TGF β -related members are initially synthesized as a prepro-protein, which is subsequently cleaved at an RXXR site to release a mature form of 110–140 amino-acid residues. In Lefty, RGKR and RQKR are found at amino-acid residues 74–77 and 132–135, respectively (Fig. 1a, b). If one of these sequences is a cleavage site, a mature protein of 291 or 233 amino acids long should be produced, which is much larger than mature forms of other TGF β -related proteins.

We have analysed the Lefty protein using the anti-Lefty antibodies α L1 and α L2 (Fig. 2). First, we transfected 293T cells⁸ with a *lefty* expression vector. Using western blot analysis, we detected a 42K protein in the transfected cells (Fig. 2). The size of this protein is consistent with that of the prepro-protein (or pro-protein). However, Lefty protein was not detected in conditioned medium. The *lefty* gene was next expressed in BALB/3T3 cells. The principal form of the Lefty protein in these cells had a relative molecular mass of 32K (Fig. 2). This value corresponds to cleavage at RGKR (amino-acid residues 74–77). On the other hand, 25K–27K as well as 32K proteins were detected in conditioned medium (Fig. 2). The size of 25K–27K corresponds to a form cleaved at RQKR (residues 132–135). Apparently, BALB/3T3 cells could secrete these processed forms. These results indicate that the Lefty prepro-protein can be differentially processed depending on the cells in which it is synthesized.

The expression of *lefty* in post-implantation mouse embryos was examined by whole-mount *in situ* hybridization (Fig. 3). In pre-primitive streak embryos at 6.0 days post-coitum (d.p.c.), *lefty* messenger RNA was undetectable (data not shown). When the primitive streak appeared (6.5 d.p.c.), *lefty* mRNA was detected in the anterior half of the streak (Fig. 3a). At 7.0 d.p.c., *lefty* is expressed along the streak except at its caudal and rostral extremities (Fig. 3b). Transverse sections made from the same embryo showed *lefty* expression in mesoderm (Fig. 3c). At this stage, there was no apparent sign of asymmetry in the expression. On 7.5 d.p.c., *lefty* expression decreased; only a low level was detectable in some mesoderm cells (data not shown). *lefty* mRNA then disappeared at the presomite headfold stage (7.75 d.p.c.; data not shown).

In embryos with a few pairs of somites (8.0 d.p.c.), *lefty* was highly expressed in two regions: the lateral region and the region near the ventral midline (Fig. 3d, e). The former region extended laterally along the anteroposterior axis, with its anterior margin caudal to the primordial heart and its posterior margin near the allantois (Fig. 3e). Surprisingly, however, expression was observed only on the left side (Fig. 3d). Transverse sections prepared from

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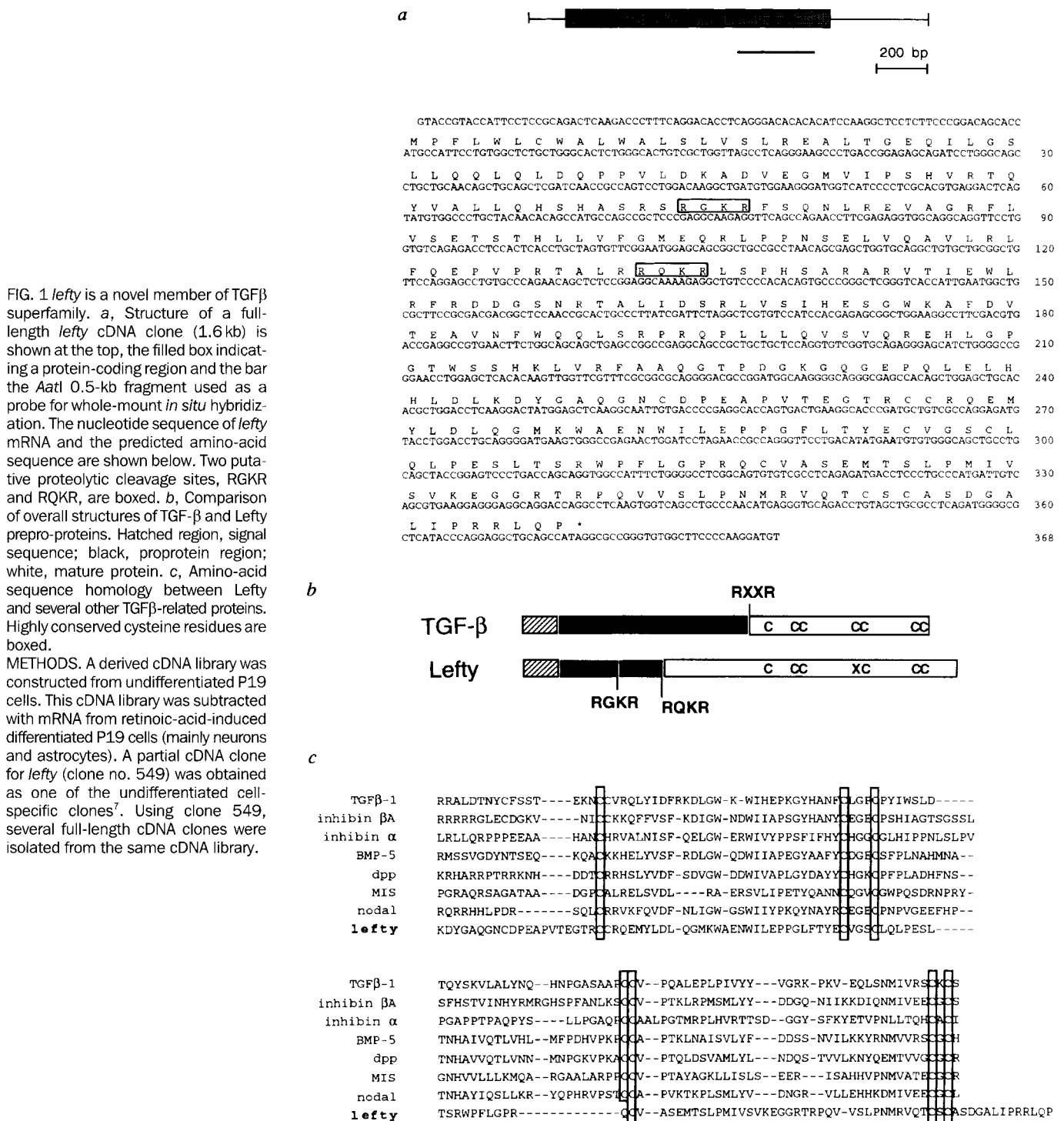


FIG. 3 Transient and asymmetrical expression of *lefty* in post-implantation embryos. *lefty* expression in various stages of mouse embryos was examined by whole-mount *in situ* hybridization. The developmental stage of each embryo was: *a*, 6.5 d.p.c.; *b* and *c*, 7.0 d.p.c.; *d* and *e*, 8.0 d.p.c., with 3–4 pairs of somites. *c*, Transverse section of a 7.0 d.p.c. embryo. *d*, Ventral view of an 8.0-d.p.c. embryo showing the asymmetrical expression of *lefty*. *e*, Lateral view (from the left) of an 8.0-d.p.c. embryo showing the extension of *lefty* expression along the anteroposterior axis. Anteroposterior (A–P) and R–L axes are indicated. *f–n*, Serial transverse sections made from an 8.0-d.p.c. embryo; seven representative sections (*f–l*) are shown. Note that *lefty* expression in the splanchnopleure is exclusively on the left side. *m*, Magnified view of the area inside the rectangle in panel *i*, emphasizing the asymmetry of *lefty* expression in the prospective floorplate. Approximate position of each section is shown in *n*. In *o*, the sites of *lefty* expression in 8.0-d.p.c. embryos are summarized: *lefty*-

expressing areas are shown in red in a ventral view of an 8.0-d.p.c. embryo. ► Abbreviations: al, allantois; ec, embryonic ectoderm; fg, foregut; lpm, lateral plate mesoderm; m, embryonic mesoderm; n, node; nf, neural fold; pfp, prospective floor plate; pmc, promyocardium; ps, primitive streak; sm, somite; sp, splanchnopleure. Scale bars, 100 µm (*b*, *e*) and 25 µm (*c*). **METHODS.** Embryos of ICR mice (Charles River) were prepared for whole-mount *in situ* hybridization according to ref. 17. To generate antisense and sense probes, the AatI 0.5-kb fragment from a cDNA clone (thick bar in Fig. 1a) was subcloned in Bluescript. Antisense and sense probes were produced with T3 and T7 RNA polymerases, respectively. The sense probe was the control in most experiments, giving no detectable staining in 6.0–9.5-d.p.c. embryos. Developmental stages were confirmed by their morphology¹⁸. In the case of embryos later than 8.0 d.p.c., the number of somites were counted. Paraffin sections were prepared (10 µm thick) after whole-mount *in situ* hybridization.

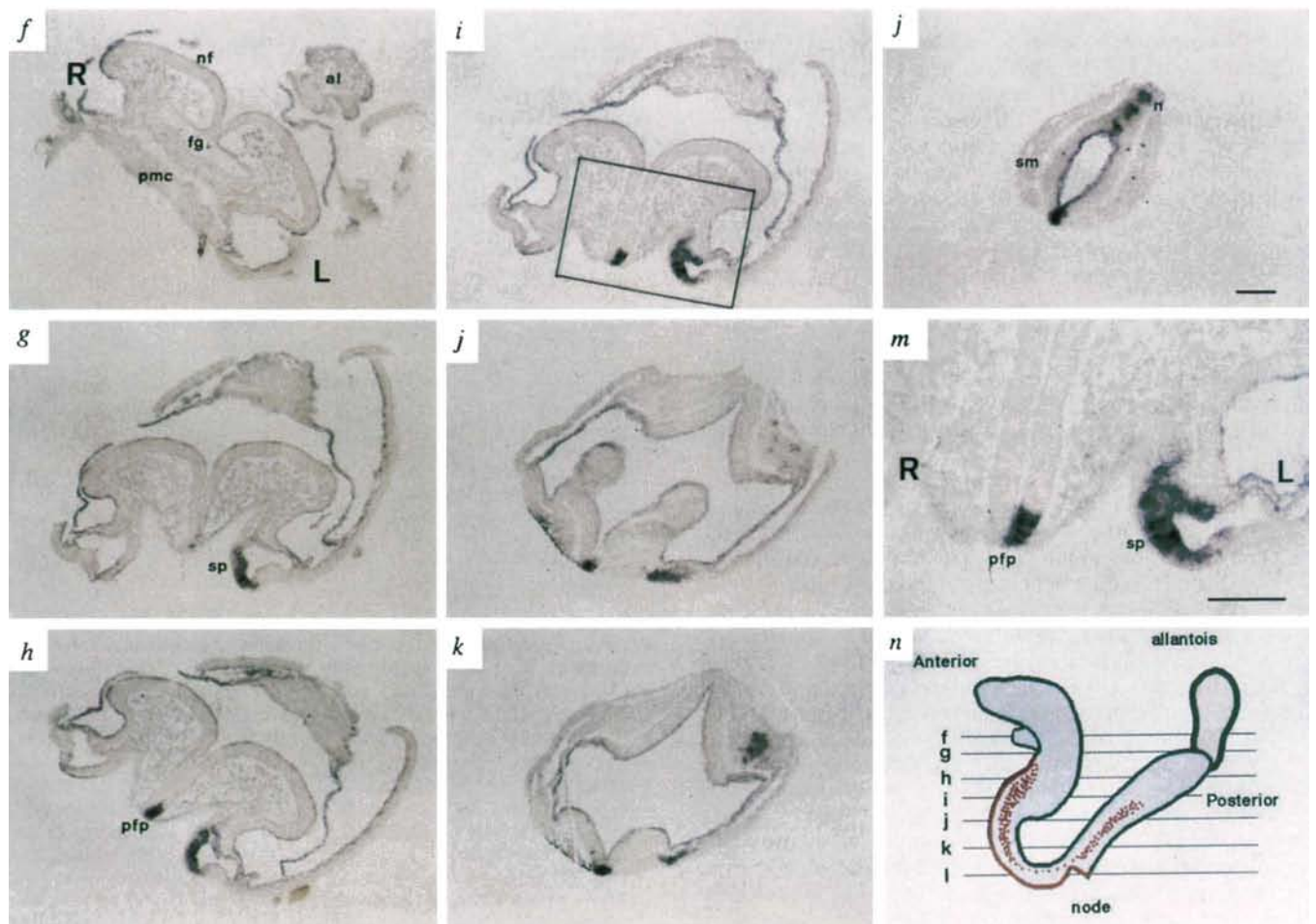
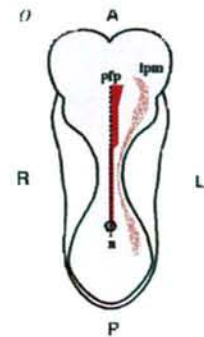
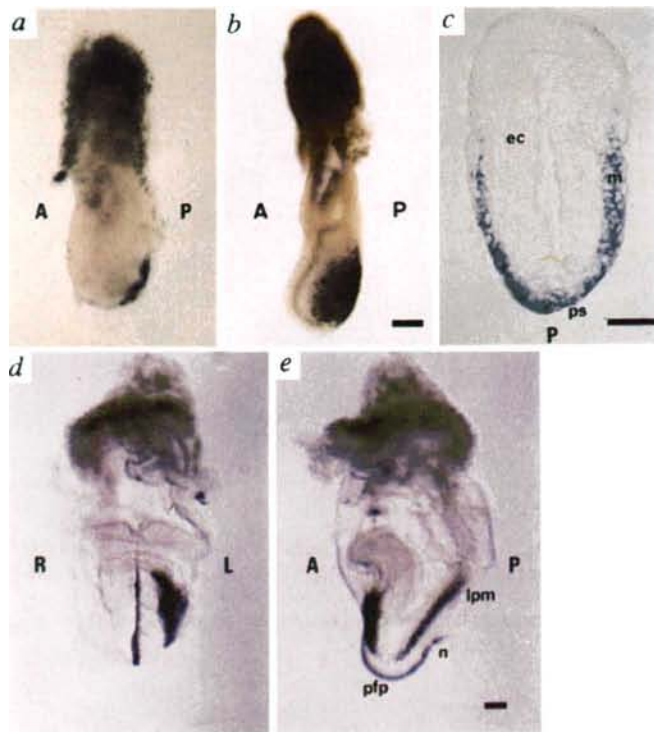
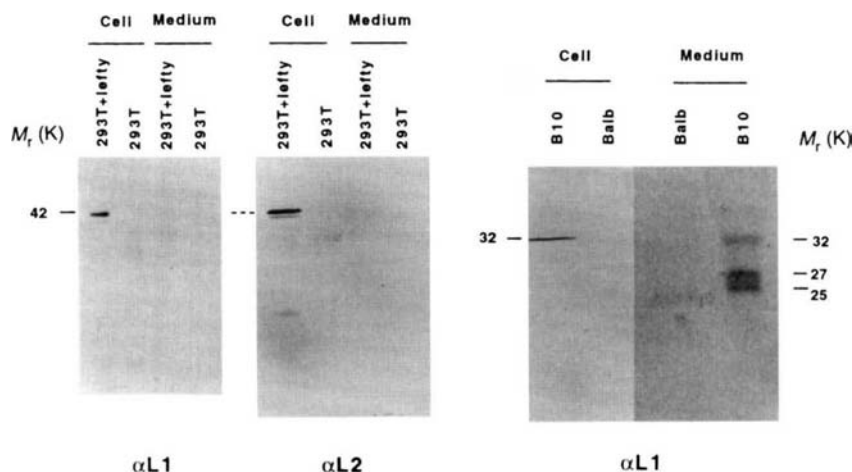


FIG. 2 Lefty protein is differentially processed according to cell type. Cell lysates and conditioned medium were prepared from the following cells: 293T cells alone, 293T cells transfected with a *lefty* expression vector (lanes 293T+*lefty*), BALB/3T3 cells (Balb) and a BALB/3T3 cell line stably transformed with a *lefty* expression vector (lanes B10). Lefty protein present in each sample was analysed by western blot with anti-Lefty antibodies, α L1 and α L2.

METHODS. Two oligopeptides, ASDGALIPRRLLQP (for α L1) and LSPHSARARVTIE (for α L2), corresponding to amino-acid residues 356–368 and 136–148, respectively, were used to immunize rabbits. Antibodies were affinity-purified and used for immunology. For ectopic expression of *lefty*, a full-length cDNA was subcloned in expression vectors pEFBOS¹⁶ and pEFSNeo (the resulting plasmids are referred to as pEFBOS-*lefty* and pEFSNeo-*lefty*, respectively). 293T cells⁸ were transfected with pEFBOS-*lefty*. 36 h after transfection, culture medium was replaced with medium containing 1% FCS. 24 h later, cells and conditioned medium were recovered for western blot analysis. Cells were suspended in PBS, sonicated, and an equal volume of 2 × SDS sampling buffer added to the sonicated solution. Proteins in conditioned medium were precipitated with trichloroacetic acid (TCA), washed with acetone and dissolved in 1 × SDS sampling buffer. *lefty*-expressing BALB/3T3 cell lines were obtained by



transfecting BALB/3T3 cells with 2.5 μ g pEFSNeo-*lefty* and 25 μ g pEFBOS-*lefty*, followed by selection with 400 μ g ml⁻¹ G418. B10, one of the stable transformants expressing a high level of Lefty protein, was mainly used here. Cell lysates and conditioned medium were prepared from BALB/3T3 or B10 cells as described for 293T cells.

8.0-d.p.c. embryos were examined (Fig. 3f–n): *lefty* was expressed in the lateral plate mesoderm (mainly splanchnopleure rather than somatopleure), but not in the promyocardium or endocardium (Fig. 3f). Therefore, *lefty*-expressing cells did not correspond to those destined to form heart.

The *lefty* gene was also expressed near the ventral midline of 8.0-d.p.c. embryos (Fig. 3d, e). Transverse sections located *lefty* expression in the prospective floorplate, but not in notochord/notochordal plate (Fig. 3f–n). Expression in the prospective floorplate was again asymmetric. In the anterior region (at the level of midbrain and hindbrain), its expression was found exclusively in the left half (Fig. 3h, i, m). The expression site gradually came closer to the midline in the more posterior region (Fig. 3j, k), and finally reached the midline in the region near the node (Fig. 3l). The *lefty*-expressing regions in an 8.0-d.p.c. embryo are all shown in Fig. 3o.

The asymmetric expression was surprisingly transient (data not shown): *lefty* mRNA was undetectable at the presomite headfold stage, appeared asymmetrically in embryos with 3–4 pairs of somites, and disappeared in embryos with 6–8 pairs of somites (8.5 d.p.c.); *lefty* expression was no longer detectable at later stages.

Two mutations in mouse, *iv*^{9,10} and *inv*¹¹, are known to cause *situs inversus*. In *iv/iv*, the L–R asymmetry seems to be randomized because only 50% of *iv* homozygotes show *situs inversus*^{9,10}. On the other hand, all *inv* homozygotes have reversed L–R polarity¹¹. To investigate the relationship between *lefty* and these mutations, we recently mapped the chromosomal location of *lefty*. The gene was located on mouse chromosome 1 (Y. Matsuda *et al.*, unpublished data). As *iv* and *inv* are located on chromosome 12 (ref. 12) and 4 (ref. 11), respectively, *lefty* is clearly different from *iv* and *inv*. We next examined *lefty* expression in *iv* and *inv* mutant embryos (Fig. 4): in about half (3/7) of the *iv/iv* embryos obtained from intercrossing *iv/iv* and *iv/iv*, *lefty* expression was inverted and found on the right side (Fig. 4a); in these embryos, the inversion of *lefty* expression was seen both in the splanchnopleure and prospective floorplate (Fig. 4b). The remaining *iv/iv* embryos (4/7) showed *lefty* expression on the left side (Fig. 4a, c). All *iv* homozygotes showed ectopic *lefty* expression in somatopleure. For *inv*, embryos were obtained from intercrossing *inv/+* and *inv/+* as homozygotes are lethal¹¹. About 1/4 of the embryos (5/17) showed *lefty* expression

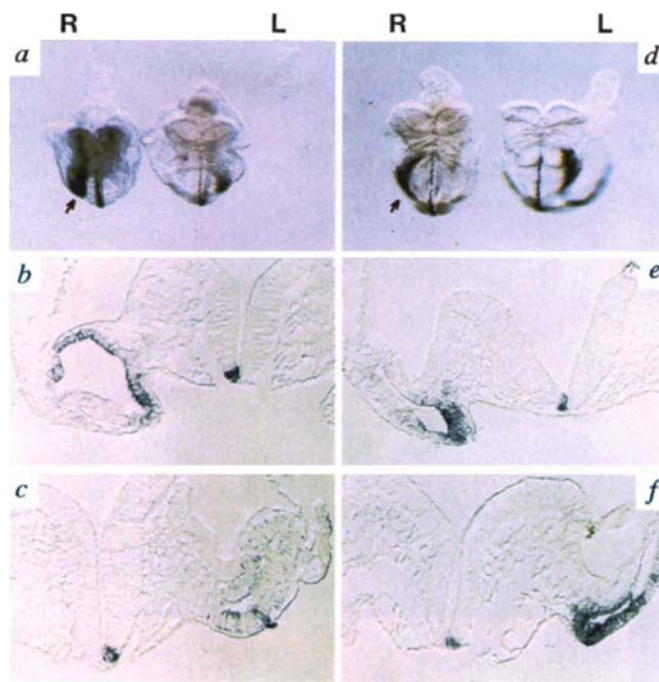


FIG. 4 Expression of *lefty* is inverted in *iv* and *inv* mutant embryos. a, b and c, Embryos from *iv/iv* × *iv/iv* intercross. d, e and f, Embryos from *inv/+* × *inv/+* intercross. The L–R axis of the embryos is indicated. a, Of seven embryos from the *iv/iv* × *iv/iv* intercross, three showed *lefty* expression on the right side (embryo with arrow), and the remaining four had expression on the left side (embryo without arrow). Transverse sections of these embryos are shown in b and c: b, embryo with inverted *lefty* expression; c, embryo with normal *lefty* expression. d, Of 17 embryos from the *inv/+* × *inv/+* intercross, five showed *lefty* expression on the right side (arrow); the remainder showed expression on the left side (no arrow). Transverse sections of these embryos are shown in e and f: e, embryo with inverted *lefty* expression; f, embryo with normal *lefty* expression. SJ/Col *iv/iv* mice (Jackson Labs) and FVB/N *inv/+* mice were used in this study.

on the right-side (Fig. 4d). In these embryos, *lefty* expression was inverted both in the splanchnopleure and prospective floorplate (Fig. 4e). The remaining embryos showed *lefty* expression on the left side (Fig. 4d,f). The inversion of *lefty* expression was not found in the embryos from the [*inv*/+ × +/+] cross (0/16), so those embryos with inverted *lefty* expression are probably *inv* homozygotes. These results show that the second wave of *lefty* expression is downstream of *iv* and *inv*.

lefty may act at a relatively late stage of L–R determination because L–R polarity in mammals is committed by the presomite stage¹³. *Lefty* protein in the splanchnopleure may attract primordial cells of heart and induce their asymmetric growth or migration. The significance of the asymmetric expression in the ventral neural tube is not clear, but it may be required for generating certain structural/functional asymmetries of the central nervous system. Asymmetric expression of activin receptor typeIIA, sonic hedgehog and *cNR-1* has been reported in chicken embryo and interpreted as representing a hierarchy of expression of these genes. The *cNR-1* gene appears to be a chicken homologue of mouse *nodal*¹⁵, and also belongs to the TGF- β superfamily. However, *lefty* is highly diverged form *cNR-1* and *nodal* according to its structure (Fig. 1c). Expression patterns of *cNR-1* and *lefty* appear similar, but there are striking differences: for example, *lefty* is expressed in one half of the prospective floorplate, a feature not shared by *cNR-1*. It is not certain whether *cNR-1* is a functional

homologue of *lefty* or whether more than one member of the TGF- β superfamily is involved in L–R asymmetry. □

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1. Brown, N. A., McCarthy, A. & Wolpert, L. in *Biological Asymmetry and Handedness* 182–201 (Wiley, Chichester, 1991).
2. Brown, N. A. & Lander, A. *Nature* **363**, 303–304 (1993).
3. McDonald, N. Q. & Hendrickson, W. A. *Cell* **73**, 421–424 (1993).
4. Kingsley, D. M. *Genes Dev.* **8**, 133–146 (1994).
5. Hogan, B. L. M. et al. *Development* suppl. 53–60 (1994).
6. McBurney, M. W. & Rogers, B. J. *Dev. Biol.* **89**, 503–508 (1978).
7. Saijoh, Y. et al. *Genes to Cells* (in the press).
8. Pear, W. S. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8392–8396 (1993).
9. Hummel, K. P. & Chapman, D. B. *J. Hered.* **50**, 9–13 (1959).
10. Layton, W. M. *J. Hered.* **67**, 336–338 (1976).
11. Yokoyama, T. et al. *Science* **260**, 679–682 (1993).
12. Brueckner, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 5035–5039 (1989).
13. Brown, N. A. & Wolpert, L. *Development* **109**, 1–9 (1990).
14. Levin, M. et al. *Cell* **82**, 803–814 (1995).
15. Zhou, X. et al. *Nature* **361**, 543–547 (1993).
16. Mizushima, S. & Nagata, S. *Nucleic Acids Res.* **18**, 5322 (1990).
17. Wilkinson, D. G. in *In Situ Hybridization: A Practical Approach* 75–84 (IRL, Oxford, 1992).
18. Theiler, K. in *The House Mouse: Atlas of Embryonic Development* 40–42 (Springer, New York, 1989).

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Relationship between asymmetric *nodal* expression and the direction of embryonic turning

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GROWTH factors related to TGF- β provide important signals for patterning the vertebrate body plan^{1–3}. One such family member, *nodal*, is required for formation of the primitive streak during mouse gastrulation^{4,6}. Here we have used a *nodal-lacZ* reporter allele to demonstrate asymmetric *nodal* expression in the mouse node, a structure thought to be the functional equivalent of the frog and chick ‘organizer’, and in lateral plate mesoderm cells. We have also identified two additional genes acting with *nodal* in a pathway determining the left–right body axis. Thus we observe in *inv* mutant embryos⁸ that the sidedness of *nodal* expression correlates with the direction of heart looping and embryonic turning. In contrast, HNF3- β ^{+/–} *nodal*^{lacZ/+} double-heterozygous embryos display LacZ staining on both left and right sides, and frequently exhibit defects in body situs. Taken together, these experiments, along with similar findings in chick⁹, demonstrate that elements of the genetic pathway that establish the left–right body axis are conserved in vertebrates.

Studies of a retrovirally induced recessive lethal mutation, termed 413.d (ref. 4), have demonstrated that *nodal*, a member of the transforming growth factor (TGF)- β superfamily of secreted growth factors, is essential for the initiation and maintenance of a distinct primitive streak^{6,10}. To document precisely the pattern of *nodal* expression, we generated a novel mutant allele (Fig. 1a) in which the second exon was replaced by an IRES sequence linked to a LacZ–neo fusion cassette¹¹ leaving the potential regulatory elements intact. Heterozygous embryos carrying the *nodal*^{lacZ} allele were morphologically normal, whereas homozygous mutants displayed exactly the same abnormalities as

those observed with the original 413.d allele (not shown). In heterozygotes, expression of β -galactosidase faithfully recapitulates that described for *nodal* transcripts (Fig. 1b). At the headfold stage we observed equivalent numbers of cells expressing *nodal* messenger RNA adjacent to the lateral edges of both sides of the developing node (Fig. 1c). In contrast, the pattern of LacZ staining extends more posteriorly, forming a continuous boundary around the caudal notochordal plate (Fig. 1d). This difference probably reflects the persistence of β -gal activity, and strongly suggests that some descendants of the *nodal*-expressing cell population are displaced caudally around the periphery of the node. By early somite stages, *nodal* mRNA expression has become markedly asymmetrical, with more intense staining consistently seen on the left (Fig. 1e). In tissue sections, approximately twice the number of LacZ-positive cells are present on the left side of the node as against the right (Figs 1f, 2c). This subtle cellular asymmetry in the mouse node is reminiscent of the transient morphological handedness of Hensen’s node in chick¹².

The increased sensitivity afforded by β -gal staining also reveals an asymmetric domain of *nodal* expression beginning at the 3–4-somite stage, confined to a subpopulation of lateral mesoderm cells on the prospective left side of the embryo (Fig. 2). This stripe of cells extends rostrally from the primitive streak to a position coincident with the caudal region of the developing heart tube (Fig. 2d). Asymmetric *nodal* expression precedes visible heart looping or axial rotation of the embryo, and persists until approximately the 12–14-somite stage, when the embryo is midway through the morphogenetic turning sequence.

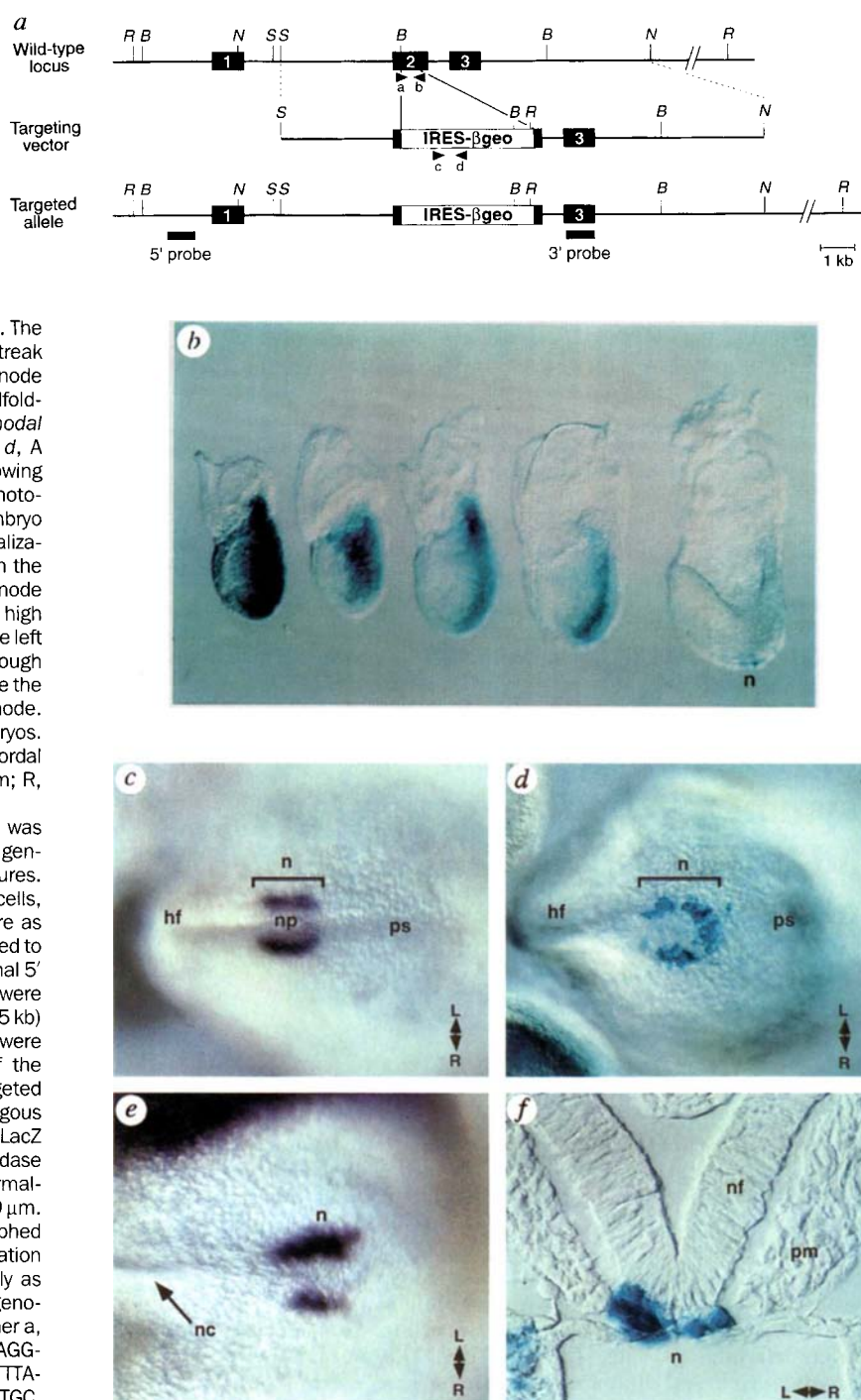
It is well established that prospective lateral plate mesoderm arises from ectodermal cells that ingress at roughly the midpoint along the proximal–distal axis of the streak^{13–18}. This cell population contributes mesoderm to both sides of the definitive body axis. In contrast, induction of *nodal-lacZ* expression in lateral mesoderm is strictly confined to the left side of the embryo. We sought to identify additional asymmetrically expressed genes that might regulate *nodal* expression. In chick, asymmetric expression of *Cnr-1* a *nodal* homologue, HNF3- β , and *sonic hedgehog* (*shh*) have been implicated in regulating left–right axis formation⁹. Moreover, ectopic *shh* expression was shown to cause alterations in the sidedness of *Cnr-1* expression and reversal of heart asymmetry⁹. The mouse *shh* gene is known to be expressed in cells of the notochordal plate and notochord^{19–21}. We found no evidence for asymmetric *shh* expression in late-streak and early-somite

FIG. 1 Introduction of a LacZ reporter gene into the mouse *nodal* locus. **a**, To generate a *nodal^{lacZ}* allele, 400 bp of coding sequence from the second coding exon of *nodal* were replaced by the IRES- β geo selection cassette¹¹. The targeting vector contained 10 kb of genomic homology. After electroporation, drug-resistant clones were screened by Southern blot analysis using external probes. About 50% of the G418^R clones were correctly targeted. Mice were genotyped by polymerase chain reaction using the indicated primers (filled triangles). Abbreviations: R, *EcoRV*; B, *Bam*HI; N, *Not*I; S, *Sac*II. **b**, Lateral views of five 7.5-d.p.c. embryos heterozygous for the *nodal^{lacZ}* allele. The LacZ staining is restricted to the region of the primitive streak in younger embryos (left) and becomes localized to the node at headfold stages (right). **c**, A ventral view of a headfold-stage embryo (7.5 d.p.c.) showing expression of *nodal* mRNA localized to the sides of the developing node. **d**, A ventral view of a headfold-stage embryo (7.5 d.p.c.) showing LacZ staining restricted to the cells surrounding the notochordal plate. **e**, Ventral view of early somite stage embryo (5–6 somites) showing pronounced asymmetrical localization of *nodal* mRNA. More intense staining is visible on the left side of the node. **f**, Transverse section through the node region of an 8-somite stage embryo. A disproportionately high number of *nodal-lacZ* expressing cells are present on the left side of the node. Cell counts performed on sections through the node region consistently showed approximately twice the number of LacZ-positive cells on the left side of the node. Similar results were obtained in 8 serially sectioned embryos. Abbreviations: ps, primitive streak; n, node; np, notochordal plate; hf, headfold; nc, notochord; ne, neural ectoderm; R, right side; L, left side of the embryo.

METHODS. A cosmid clone spanning the *nodal* locus was isolated and appropriate genomic fragments used to generate the targeting vector according to standard procedures. Conditions for electroporation of CCE embryonic stem cells, G418 selection and generation of chimaeric mice were as described²⁴. For Southern blot analysis, DNA was digested to completion with *Bam*HI and filters probed with an external 5' genomic probe as indicated. Correctly targeted clones were identified by the presence of diagnostic wild-type (6.5 kb) and mutant (12.5 kb) fragments. Selected samples were hybridized with the 3' probe to verify the fidelity of the recombination event. Six independent correctly targeted clones produced germline chimaeras, and heterozygous progeny of all of these showed the same pattern of LacZ staining. Intact embryos were stained for β -galactosidase activity as described²⁵, and then postfixed in 4% paraformaldehyde, embedded in paraffin wax and sectioned at 9 μ m. Sections were mounted in 80% glycerol and photographed under Nomarski optics. Whole mount *in situ* hybridization using a *nodal*-specific probe was performed essentially as described²⁶. The sequence of the primers used for genotyping animals was as follows: for wild-type allele, primer a, ATGTGGACGTGACCGACAGAACT, primer b, CTGGATGTAGG-CATGGTTGGTAGGAT; for mutant allele, primer c, GTCGTTTCAACGTCGTGACT, primer d, GATGGGCGCATCGTAACCGTGC.

stage embryos by analysing either mRNA expression or using shh antibodies²¹ (not shown).

To describe the possible role of *nodal* signalling during left-right axis formation, we used a recessive insertional mutation termed *inv*, which reverses the direction of embryonic turning, causing a reversal of body situs⁸. We mated *inv*^{+/+} female mice to males heterozygous for both *inv* and *nodal^{lacZ}*, and progeny were recovered at about 8.5 days of gestation. About 20% of the embryos displayed reversal of the direction of axial rotation. As shown in Fig. 3, *nodal-lacZ* expression in lateral mesoderm in these presumed *inv* homozygotes was confined to the prospective right-hand side of the embryo. Thus there is a close correlation between the sidedness of *nodal* expression and the direction of embryonic turning. In two of the seven *inv/inv* mutant embryos examined, the LacZ staining appeared as a precise mirror-image duplication of the wild-type pattern, with LacZ-positive cells



extending into the pericardial cavity (Fig. 3b). The remaining embryos showed a rostral truncation of the *nodal-lacZ* expression domain below the level of the pericardial cavity (Fig. 3a). This truncation was associated with a random or ambiguous direction of heart looping (Fig. 3c, d) and may account for similar observations noted in ref. 8.

HNF3- β , a member of the HNF3/*fork head* family of transcription factors, is essential for mouse gastrulation and node formation^{22,23}. To determine whether HNF3- β and *nodal* act together in the pathway determining left-right asymmetry, females carrying a null mutation at the HNF3- β locus²² were crossed with HNF3- β ^{+/+} males carrying the *nodal^{lacZ}* allele. There was no *nodal-lacZ* expression detectable in HNF3- β homozygous mutants at 8.5 days post-coitum (d.p.c.; data not shown). Strikingly, all of the HNF3- β ^{+/+} embryos showed bilateral LacZ staining in lateral plate mesoderm (Fig. 4). Most of these double-heterozygous

embryos showed stronger staining on the prospective left side, and exhibited normal turning. In contrast, in embryos showing equivalent *nodal-lacZ* expression on both left and right sides, the direction of turning was random (Fig. 4b). The onset of turning was noticeably delayed in all of the double heterozygotes compared to their littermates. Additionally, in most instances the *nodal-lacZ* expression domains fail to extend into the pericardial cavity (Fig. 4c). To further describe functional consequences of *nodal* misexpression, litters were dissected at 15.5 d.p.c. All ten $\text{HNF3-}\beta^{+/-} \text{nodal}^{+/+}$ embryos were grossly normal, whereas seven of ten $\text{HNF3-}\beta^{+/-} \text{nodal}^{lacZ/+}$ double heterozygotes showed marked defects in the positioning of the abdominal viscera and

the heart. These findings demonstrate a genetic interaction between *HNF3-}\beta* and *nodal* in establishment of left-right asymmetry. However, *HNF3-}\beta* mRNA does not appear to be asymmetrically expressed in mouse embryos (not shown). It therefore remains unclear which site of *HNF3-}\beta* expression, namely the visceral endoderm or the developing node²², regulates asymmetric *nodal* expression at later stages of embryogenesis.

The essential role of *nodal* during gastrulation can be attributed to its early site of expression in the embryonic ectoderm. Here we show that *nodal* is asymmetrically expressed in two non-overlapping cell populations in late-streak-early-somite stage embryos. These are the ventral-lateral cells of the mature node

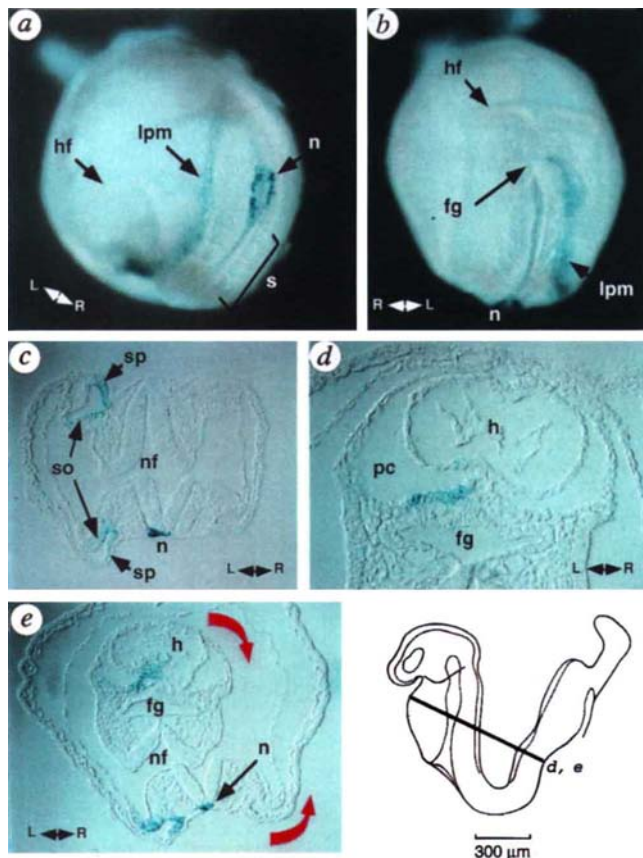
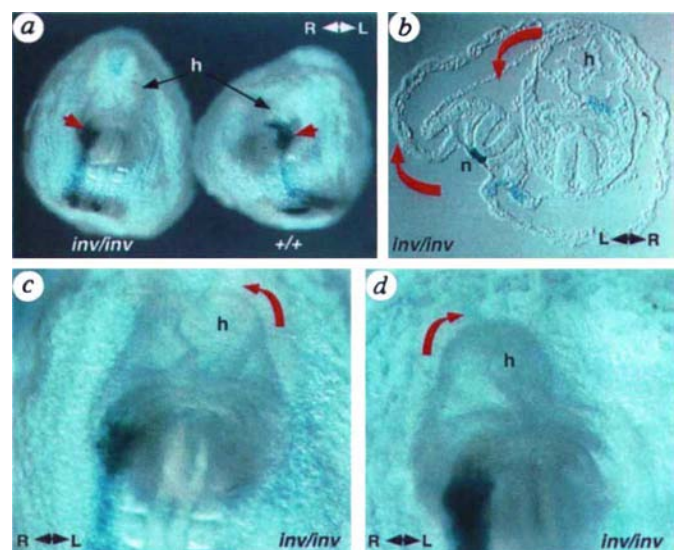


FIG. 2 Asymmetric *nodal-lacZ* expression in lateral plate mesoderm and in the developing heart. **a, b**, Ventral and frontal views, respectively, of a 4-somite stage heterozygous embryo stained for β -gal activity. In addition to the mature node, *nodal* expression was observed in a band of cells present on the left side of the embryo, with a rostral limit at the position of the forming heart. **c, d**, Transverse sections through a 5–6-somite stage embryo before turning. Left-sided *nodal* expression was detectable in both the somatic and splanchnic mesoderm. **d**, Higher-magnification view of the heart region showing asymmetric *nodal* expression before overt heart looping or axial rotation. **e**, Transverse section through a 7–8 somite stage embryo showing *nodal* expression extending into the dorsal wall of the pericardial cavity adjacent to the myocardial layer. The dorsal tube is visibly asymmetrical owing to looping and the embryo has initiated the turning process. The direction of axial rotation of the cephalic and caudal regions is normally to the right (indicated by the red arrows) and thus away from the *nodal* expression domain. The panel illustrating the plane of section is adapted from ref. 27. As a control, at all stages examined, *snail* mRNA, a second gene expressed in lateral plate mesoderm^{28,29}, is expressed at equivalent levels on both sides (not shown). Abbreviations: fg, foregut; h, heart; hf, headfold; lpm, lateral plate mesoderm; n, node; nf, neural fold; pc, pericardial cavity; so, somatic mesoderm; sp, splanchnic mesoderm; s, somite; R, right side; L, left side of the embryo.

FIG. 3 Correlation between the sidedness of *nodal* expression and the direction of embryonic turning in *inv* mutant embryos. Mutant embryos from 8 to 12 somite stages were collected and stained for β -gal activity. **a**, Frontal view of a presumed *inv* homozygous mutant (left) and a wild-type or heterozygous littermate (right) showing *nodal* expression in lateral plate mesoderm confined to the prospective right and left sides, respectively. In this *inv* mutant embryo, and in contrast to wild-type embryos, the rostral limit of the *nodal-lacZ* expression domain does not extend anteriorly into the pericardial cavity (red arrowheads). **b**, Transverse section through an *inv* mutant embryo exhibiting asymmetric *nodal* expression on the right side that extends into the dorsal wall of the pericardial cavity. The embryo is turning towards the left. **c**, Higher magnification of the *inv* mutant embryo shown in **a**. **d**, A second mutant embryo showing a rostral truncation in the *nodal* expression domain. The hearts of these two mutant embryos loop in opposite directions (indicated by red arrows). R, right side; L, left side of the embryo.

METHODS. Crosses were established between double heterozygous *inv* $^{+/-}$, *nodal*^{lacZ/+} males and *inv* $^{+/-}$ females. Embryos were collected at 8.5 d.p.c. and stained for β -gal activity.



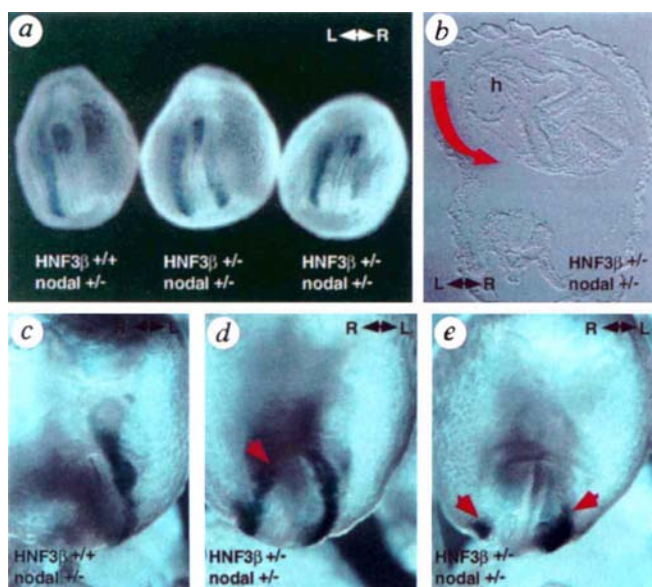


FIG. 4 Misexpression of *nodal* in *HNF3-β*^{+/-} *nodal*^{lacZ}^{+/-} double heterozygotes. Embryos were collected at the 8–12-somite stage and stained for β-gal activity. **a**, Posterior views of two *HNF3-β*^{+/-} *nodal*^{lacZ}^{+/-} embryos (right) and a *nodal*^{lacZ}^{-/-} littermate (left) showing bilateral and left-sided LacZ staining, respectively. Note the differences in the intensity and extent of LacZ staining between these two double heterozygotes. **b**, Transverse section through an *HNF3-β*^{+/-} *nodal*^{lacZ}^{+/-} heterozygous embryo showing bilateral LacZ expression associated with a reversal in the direction of turning. The plane of section coincides with the extreme rostral limit of the LacZ staining domain. **c–e**, Frontal views of the embryos shown in **a**, to illustrate the rostral truncation of the *nodal*–*lacZ* expression domain observed in most of the double heterozygotes.

METHODS. Crosses were established between *HNF3-β*^{+/-} *nodal*^{lacZ}^{-/-} males and *HNF3-β*^{+/-} females. Embryos were collected at 8.5 d.p.c. and stained for β-gal activity.

immediately adjacent to the notochordal plate, and a discrete subpopulation of mesodermal cells on the left side of the embryo originating from more proximal regions of the streak. Moreover, the sidedness of *nodal* expression in lateral plate mesoderm predicts the direction of axial rotation and heart looping. Misexpression of *nodal* leads to ambiguities in turning, heart situs and the polarity of the viscera. Thus it seems likely that *nodal* signalling is responsible for highly localized effects on cell migration or proliferation necessary to promote these morphogenetic movements. The *nodal*–*lacZ* reporter mice described here should allow further evaluation of genetic interactions with other molecular components specifying body situs in vertebrates. □

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- Kessler, D. S. & Melton, D. A. *Science* **266**, 596–604 (1994).
- Kingsley, D. M. *Genes Dev.* **8**, 133–146 (1994).
- Conlon, F. L. & Beddington, R. S. P. *Semin. dev. Biol.* **6**, 249–256 (1995).
- Conlon, F. L., Barth, K. S. & Robertson, E. J. *Development* **111**, 969–981 (1991).
- Iannaccone, P. M., Zhou, X., Khokha, M., Boucher, D. & Kuehn, M. R. *Dev. Dyn.* **194**, 198–208 (1992).
- Conlon, F. L. *et al.* *Development* **120**, 1919–1928 (1994).
- Beddington, R. S. P. *Development* **120**, 613–620 (1994).
- Yokoyama, T. *et al.* *Science* **260**, 679–682 (1993).
- Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. & Tabin, C. *Cell* **82**, 803–814 (1995).
- Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L. M. & Kuehn, M. R. *Nature* **361**, 543–547 (1993).
- Mountford, P. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **91**, 4303–4307 (1994).
- Cooke, J. *Nature* **374**, 681 (1995).
- Beddington, R. S. P. *J. Embryol. exp. Morph.* **64**, 87–104 (1981).
- Beddington, R. S. P. *J. Embryol. exp. Morph.* **69**, 265–285 (1982).
- Lawson, K. A., Meneses, J. J. & Pedersen, R. A. *Development* **113**, 891–911 (1991).
- Tam, P. P. L. & Beddington, R. S. P. *Development* **99**, 109–126 (1987).
- Smith, J. L., Gesteland, K. M. & Schoenwolf, G. C. *Dev. Dyn.* **201**, 279–289 (1994).
- Parameswaran, M. & Tam, P. P. L. *Dev. Genet.* **17**, 16–28 (1995).
- Echelard, Y. *et al.* *Cell* **75**, 1417–1430 (1993).
- Chang, D. T. *et al.* *Development* **120**, 3339–3353 (1994).
- Marti, E., Takada, R., Bumcrot, D. A., Sasaki, H. & McMahon, A. P. *Development* **121**, 2537–2547 (1995).
- Ang, S.-L. & Rossant, J. *Cell* **78**, 561–574 (1994).

- Weinstein, D. C. *et al.* *Cell* **78**, 575–588 (1994).
- Poirier, F. & Robertson, E. J. *Development* **119**, 1229–1236 (1993).
- Hogan, B., Beddington, R., Costantini, F. & Lacy, E. *Manipulating the Mouse Embryo: A Laboratory Manual*. (Cold Spring Harbor Laboratory Press, NY, 1994).
- Wilkinson, D. G. in *In situ Hybridization* (ed. Wilkinson, D. G.) 75–83 (IRL, Oxford, 1992).
- Kaufman, M. H. *Atlas of Mouse Development* (Academic, London, 1992).
- Neito, M. A., Bennett, M. F., Sargent, M. G. & Wilkinson, D. G. *Development* **116**, 227–237 (1992).
- Smith, D. E., Del Amo, F. F. & Gridley, T. *Development* **116**, 1033–1039 (1992).

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Conserved left–right asymmetry of nodal expression and alterations in murine *situs inversus*

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VERTEBRATES have characteristic and conserved left–right (L–R) visceral asymmetries, for example the left-sided heart. In humans, alterations of L–R development can have serious clinical implications, including cardiac defects¹. Although little is known about how the embryonic L–R axis is established, a recent study in the chick embryo revealed L–R asymmetric expression of several previously cloned genes, including *Cnr-1* (for *chicken nodal-related-1*), and indicated how this L–R molecular asymmetry might be important for subsequent visceral morphogenesis². Here we show that *nodal*³ is asymmetrically expressed in mice at similar stages, as is *Xnr-1* (for *Xenopus nodal related-1*)⁴ in frogs. We also examine *nodal* expression in two mouse mutations that perturb L–R development, namely *situs inversus viscerum* (*iv*)⁵, in which assignment of L–R asymmetry is apparently random and individuals develop either normally or are mirror-image-reversed (*situs inversus*), and *inversion of embryonic turning* (*inv*)⁶, in which all individuals develop with *situs inversus*. In both, *nodal* expression is strikingly affected, being reversed or converted to symmetry. These results further support a key role for *nodal* and *nodal*-related genes in interpreting and relaying L–R patterning information in vertebrates. To our knowledge, our results provide the first direct evidence that *iv* and *inv* normally function well before the appearance of morphological L–R asymmetry.

In the chick embryo, *Cnr-1* is expressed on the left side of Hensen's node beginning at stage 6, but becomes symmetric by stage 9. *Cnr-1* is also found in the left lateral-plate mesoderm (LPM) from stages 8 to 11 (ref. 2). In the mouse embryo, *nodal* expression had previously been studied only at stages before and during early mesoderm formation^{3,7}. In contrast to the chick, pre-somite-stage mouse embryos show symmetric *nodal* expression around the mouse node (equivalent to Hensen's node). Using whole-mount

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in situ hybridization, we examined mouse embryos at stages equivalent to those in chick development when *Cnr-1* is asymmetrically expressed in the LPM. Our analysis revealed that *nodal* expression is asymmetric in the mouse embryo, both around the node and in the LPM. Expression becomes stronger on the left side of the node (Fig. 1*a-c*), a sidedness identical to that shown by *Cnr-1* expression in the chick. Asymmetry was seen as early as the 2–3-somite stage, but a strict correlation between developmental stage and onset of asymmetry is not evident (Table 1). Expression in the left LPM, detected as early as 2–3 somites in a small fraction of mouse embryos, is evident in most embryos by 5–6 somites, but is no longer seen after 9–10 somites (Table 1*a* and Fig. 1). Over this brief period, expression expands from a small region just lateral to the node (Fig. 1*d*), to include the entire LPM (Fig. 1*e,h*), then becomes restricted to anterior LPM (Fig. 1*f*), but is not detected in the heart (Fig. 1*g*). We also examined post-gastrulation expression of the three *Xenopus nodal*-related genes^{4,8}. Only *Xnr-1* transcripts are detected at these stages, appearing first in a bilaterally symmetric domain flanking the posterior limit of the notochord (Fig. 2*a*), then in the left LPM (Fig. 2*b,f*). Spatial changes in the LPM domain of *Xnr-1* expression occur (Fig. 2*c-e,g*) that are similar to those seen for *nodal* in the mouse embryo. Whereas there are differences in the expression patterns of *nodal*, *Cnr-1* and *Xnr-1* around the node and notochord in mice, chicks and frogs, the conserved asymmetric LPM expression in these vertebrates suggests that these are cognate genes that might have similar functions in L–R determination.

The best characterized mouse mutations affecting L–R development are *iv* and *inv*.

TABLE 1 Correlation of asymmetric *nodal* expression and developmental age in normal and mutant mouse embryos

(a) Normal embryos		Somite numbers									
		0-1	1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Node											
Symmetric		5	5	4	4	4	5	3	1	1	0
Asymmetric		0	4	2	4	9	5	5	8	4	2
No expression									2	8	5
Per cent asymmetric		0	44	33	50	69	50	63	89	80	100
Lateral plate											
No expression		5	7	5	2	1	0	1	8	11	7
Left-side expression		0	2	1	6	12	10	7	3	2	0
Per cent expressing		0	22	17	75	92	100	88	27	15	0

(b) Mutant embryos		Somite numbers							
		3-4	4-5	5-6	6-7	7-8	8-9		
inv/+ × inv/+	N*	L†	L†	L†	L† L‡	R* N†	N† N‡		
	N†	L‡	L‡	L‡	L† R*	L† N†	N† N‡		
	N†	R*	R*	R*	L† N†	N† N‡	N† N‡		
				R†§	L† N†	N†			
iv/iv × iv/iv	N	N R	N L	N B	N B	N B	N		
	N	N B	N R	L B	L B	L B			
	R	L B	L R	L B	L B	R B			
	B		L	R B	R B				

In *a*, a compilation of the *nodal* expression pattern of 90 wild-type embryos at different developmental stages (indicated by the number of somites) is shown. Embryos were scored for the pattern of expression around the node and for the presence of LPM expression. For each age group, those showing asymmetric expression around the node are compared to the total with expression around the node, and those showing expression in the left lateral plate are compared to the overall total. Each comparison is given as a percentage. In *b*, the pattern of *nodal* expression in the LPM of individual embryos of different developmental stages and different genotypes is shown. The genotype (if known) of embryos from the *inv/+* by *inv/+* cross is indicated. All embryos from the *iv/iv* by *iv/iv* cross are homozygous. N, no lateral plate expression detected; L, left lateral plate expression; R, right lateral plate expression; B, expression in both lateral plates.

* Genotyped by PCR as *inv/inv*.

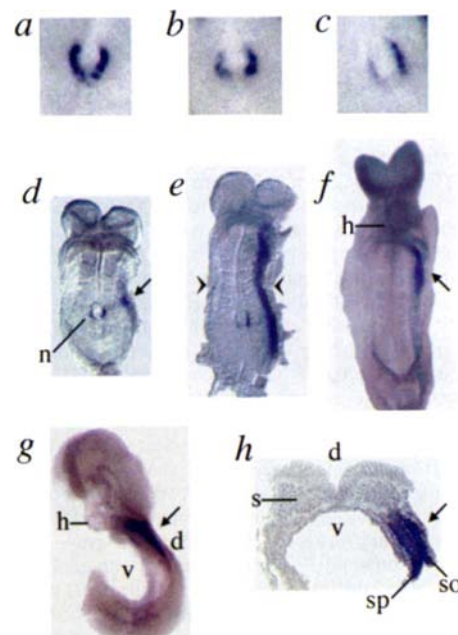
† Genotyped as *inv/+* or *+/+*.

‡ Unknown genotype (PCR failure).

§ Apparent genotyping error.

FIG. 1 Analysis of *nodal* mRNA expression in wild-type mouse embryos. In *a–f*, embryos are oriented with anterior to the top and are viewed from the ventral side. Therefore each embryo's left side is toward the right in the figure. Progression to asymmetry around the node is illustrated in close-ups of *a*, a pre-somite embryo; *b*, a 3–4-somite embryo; and *c*, a 5–6-somite embryo. *d–f*, Changing pattern of expression in the lateral-plate mesoderm: *d*, a 4–5-somite embryo with limited expression lateral to the node (arrow); *e*, a 5–6-somite embryo with expression throughout the lateral plate; *f*, a 7–8-somite embryo with mostly anterior expression (arrow). In *g*, a left-side view of a 7–8-somite embryo illustrates that lateral plate expression (arrow) does not extend into the heart (*h*). In *h*, a cross-section (magnification, $\times 80$) from a 5–6-somite embryo cut at the level marked (arrowheads) in *e*, shown with its left side towards the right of the figure, indicates expression in both splanchnopleure (*sp*) and somatopleure (*so*). Other abbreviations: *d*, dorsal; *n*, node; *s*, somite; *v*, ventral.

METHODS. Gestational-day-eight embryos (BALB/c by Sw) were dissected free of extra-embryonic membranes to lessen lordosis, then fixed and processed for whole-mount *in situ* hybridization as described²², with modifications. Embryos were hybridized in 50% formamide, 0.75 M NaCl, 10 mM PIPES (pH 6.8), 1 mM EDTA, 0.1 mg ml⁻¹ tRNA, 0.1% BSA, 1% SDS and 0.1% Tween-20, and washed in TBST²³; goat serum was used instead of sheep serum. BM-Purple (Boehringer Mannheim) was used for the colour reaction. The *nodal* antisense probe has been described³. Following hybridization, embryos were embedded in plastic and cross-sectioned at 10 microns.



The *iv* mutation results in approximately half of *iv/iv* mice developing with *situs inversus*. In comparison, almost all *inv* mutants show *situs invertus*. We examined *iv* and *inv* mutant embryos at appropriate developmental stages to determine whether these mutations affect the asymmetric expression of *nodal*. For *inv*, because of post-natal lethality of homozygotes, we examined embryos from crosses between heterozygotes and identified *inv* homozygotes using polymerase chain reaction (PCR). Interestingly, *inv* homozygotes consistently express *nodal* on the opposite side from normal, in the right LPM (Fig. 3a and Table 1b). Expression around the node was either symmetric or stronger on the right in most homozygotes. However, one did show discordance of node and LPM asymmetry (Fig. 3a). The pattern of *nodal* expression in *iv* embryos was complex. Although some had normal expression in the left LPM, most showed altered patterns of expression. These included right LPM expression, expression of *nodal* on both sides, and the complete lack of LPM expression (Fig. 3b, c and Table 1b). Several embryos without detectable expression had between 4 to 6 somites, in the middle of the predicted expression period. No discordance of node and LPM asymmetry was observed in *iv* mutants.

Asymmetric expression of *nodal* provides a valuable L-R marker for understanding the time and mode of action of *iv* and *inv*. Both genes, identified by their effects on visceral *situs* in mice, clearly act upstream of *nodal* and well before the appearance of the first morphological asymmetry. The *iv* mutation, as well as other mutations in mice^{9,10} and humans¹¹ produce randomization of L-R asymmetric structures. A number of experimental embryo manipulations lead to similar effects¹²⁻¹⁶. The finding that the generation of asymmetry can be genetically or experimentally separated from the assignment of a specific handedness has suggested that there are distinct mechanisms for each^{5,17-19}.

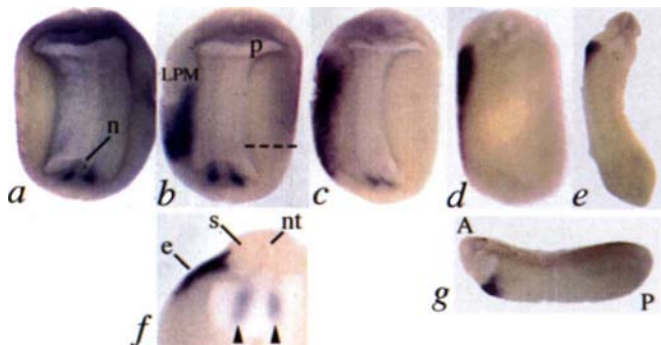


FIG. 2 Analysis of *Xnr-1* mRNA expression in neurula and early tailbud *Xenopus* embryos. In a-e, whole-mount stained embryos (nominally, stages 18, 19/20, 21, 22 and 25), were cleared after post-fixing and are viewed dorsally, with anterior oriented to the top of the page. Therefore each embryo's left side is towards the left in the figure. *Xnr-1* expression is down-regulated following gastrulation⁴, but reappears during neurulation, first as two bilaterally symmetric patches adjacent to the notochord (n), beginning at stage 18 (a) and disappearing after stage 21 (c). A larger domain of *Xnr-1* expression appears in the left LPM from stage 19, first localized in posterior/dorsal mesoderm, and then spreading anteriorly to cover the majority of the LPM (c). Subsequently (d, e), *Xnr-1* expression becomes restricted to an anterior/ventral region just posterior of the presumptive heart primordium, adjacent to the developing posterior foregut. In f, the embryo in b was bisected (as indicated by the broken line), and photographed from an anterior aspect, focusing on the *Xnr-1* signal in the splanchnopleure and somatopleure layers of the LPM. Arrowheads indicate the out-of-focal-plane bilateral expression in the posterior mesoderm. In g, a lateral view of the embryo in e is shown to highlight the ventral *Xnr-1* expression. Abbreviations: A, anterior; P, posterior; e, epidermis; LPM, lateral plate mesoderm; n, notochord; nt, neural tube; p, pharyngeal cavity; s, somite. METHODS. Whole-mount *in situ* hybridization with a full-length antisense *Xnr-1* RNA probe generated from the entire cDNA⁴ was carried out essentially as described²⁴ with minor modifications, including the use of BM-Purple and embryo powder as an additional blocking reagent to reduce background.

Based on the *iv* phenotype, it has been assumed that the gene's normal function is to bias handedness after asymmetry is established. Therefore, we predicted that half of *iv* mutant embryos would express *nodal* on the left and half on the right. However, more than 50 per cent show bilaterally symmetric expression or no expression at all, suggesting that *iv* also functions in the breaking of initial L-R symmetry. Thus, although these processes are separable, their regulation may be intimately linked. We speculate that *iv* embryos with bilateral or lacking LPM expression, may be similar to experimentally manipulated chick embryos in which *Cnr-1* is bilateral or absent², and undergo normal but randomly directed heart looping. However, subsequent L-R developmental abnormalities in these embryos might explain the significant fraction of *iv* mice displaying isomerisms and heterotaxia, namely discordance of the sidedness of different organs, such as normal heart *situs* but an inverted stomach and spleen^{5,20,21}. In *inv* mutants, *nodal*'s consistent right LPM expression correlates with the high incidence of complete *situs invertus* seen in older *inv* embryos and newborn mice⁶. A small fraction of *inv* mice show

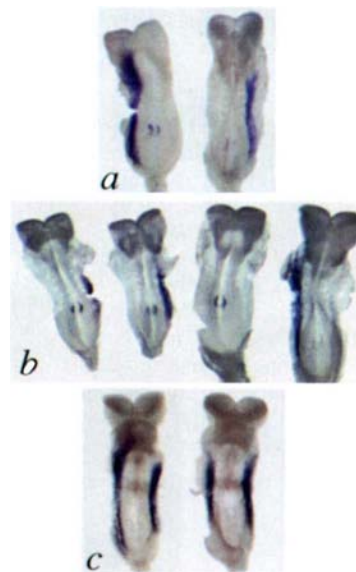


FIG. 3 Analysis of *nodal* mRNA expression in *inv* and *iv* mouse embryos. In a and b, embryos are viewed dorsally, with anterior oriented to the top of the page. Therefore each embryo's left side is towards the left in the figure. In a, two 5-6-somite embryos from an *inv/+* by *inv/+* cross are shown. The embryo with right lateral plate expression is an *inv/inv* homozygote. In b, four *iv/iv* embryos are shown: two 6-7-somite embryos with right lateral plate expression; a 3-4-somite embryo lacking lateral plate expression; and a 7-8-somite embryo with normal left lateral plate expression. In c, two 6-7-somite *iv/iv* embryos are viewed ventrally (each embryo's left side is towards the right in the figure). Each shows expression of *nodal* in both the right and the left lateral plates. Note that the extent of expression in the right and left LPM differs in these two embryos, indicating that a degree of L-R asymmetry exists even though these are overtly symmetric for *nodal* expression. METHODS. Female mice heterozygous for the *inv* mutation were super-ovulated and mated to male heterozygotes. Embryos were collected and processed for *in situ* hybridization as described for wild-type embryos. Following hybridization, individual embryos were lysed, their DNA purified and PCR used to amplify either a fragment of the tyrosinase transgene or a fragment of the region deleted in the *inv* mutation. After PCR, reactions were gel-electrophoresed, Southern blotted and hybridized to radioactively labelled probes corresponding to each amplified fragment. Embryos that showed amplification only of the transgene were genotyped as *inv/inv*; all others were genotyped as wild-type or heterozygous. For *iv*, embryos were collected from superovulated SI/COL females mated with SI/COL males or from natural matings of SI/COL animals, and processed as described for wild-type embryos.

heterotaxia. A molecular basis for this might be the discordance of node and LPM asymmetry that was noted in one individual. A low frequency of abnormal expression in the LPM, similar to that seen in *iv* embryos, might also be possible but not yet seen owing to our small sample size.

Determining how *iv* and *inv* normally regulate *nodal*'s non-random asymmetric expression awaits the isolation of these genes, and will be of key importance to the study of L–R development. *Nodal*'s role in subsequent asymmetric morphogenesis must also be established. The evolutionary conservation of *nodal*'s L–R asymmetry supports the hypothesis that this molecule is a critical component of the signalling cascade in all vertebrates that culminates in L–R morphological asymmetries. The expression patterns of *nodal* and *Xnr-1* show a remarkable similarity to each other and to *Cnr-1*, suggesting that a basic genetic framework for L–R patterning has been evolutionarily conserved in vertebrates that have quite different mechanisms of gastrulation. □

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1. Burn, J. *Ciba Found. Symp* **162**, 282–296 (1991).
2. Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. & Tabin, C. *Cell* **82**, 803–814 (1995).
3. Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L. & Kuehn, M. R. *Nature* **361**, 543–547 (1993).
4. Jones, C. M., Kuehn, M. R., Hogan, B. L. M., Smith, J. C. & Wright, C. V. E. *Development* **121**, 3651–3662 (1995).
5. Layton, W. J. *J. Hered.* **67**, 336–338 (1976).
6. Yokoyama, T. et al. *Science* **260**, 679–682 (1993).
7. Conlon, F. L. et al. *Development* **120**, 1919–1928 (1994).
8. Smith, W. C., McKendry, R., Ribisi, S. J. & Harland, R. M. *Cell* **82**, 37–46 (1995).
9. Metzler, M. et al. *EMBO J.* **13**, 2056–2065 (1994).
10. van der Hoeven, F. et al. *Development* **120**, 2601–2607 (1994).
11. Pansera, F. *Med. Hypoth.* **42**, 283–284 (1994).
12. Yost, H. J. *Development* **110**, 865–874 (1990).
13. Fujinaga, M. & Baden, J. M. *Teratology* **44**, 453–462 (1991).
14. Yost, H. J. *Nature* **357**, 158–161 (1992).
15. Fujinaga, M., Hoffman, B. B. & Baden, J. M. *Dev. Biol.* **162**, 558–567 (1994).
16. Danos, M. C. & Yost, H. J. *Development* **121**, 1467–1474 (1995).
17. Brown, N. A. & Wolpert, L. *Development* **109**, 1–9 (1990).
18. Klar, A. J. *Trends Genet.* **10**, 392–396 (1994).
19. Yost, H. J. *Cell* **82**, 689–692 (1995).
20. Brown, N. A., Hoyle, C. I., McCarthy, A. & Wolpert, L. *Development* **107**, 637–642 (1989).
21. Seo, J. W., Brown, N. A., Ho, S. Y. & Anderson, R. H. *Circulation* **86**, 642–650 (1992).
22. Henrique, D. et al. *Nature* **375**, 787–790 (1995).
23. Hogan, B., Beddington, R., Costantini, F. & Lacy, E. *Manipulating the Mouse Embryo, a Laboratory Manual* 2nd edn 357–362 (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1994).
24. Harland, R. M. *Meth. Cell Biol.* **36**, 685–695 (1991).

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Perceived visual speed constrained by image segmentation

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LITTLE is known about how or where the visual system parses the visual scene into objects or surfaces. However, it is generally assumed that the segmentation and grouping of pieces of the image into discrete entities is due to 'later' processing stages, after the 'early' processing of the visual image by local mechanisms selective for attributes such as colour, orientation, depth, and motion¹. Speed perception is also thought to be mediated by early mechanisms tuned for speed^{2–5}. Here we show that manipulating the way in which an image is parsed changes the way in which local speed information is processed. Manipulations that cause multiple stimuli to appear as parts of a single patch degrade speed discrimination, whereas manipulations that per-

ceptually divide a single large stimulus into parts improve discrimination. These results indicate that processes as early as speed perception may be constrained by the parsing of the visual image into discrete entities.

We previously measured the human ability to combine speed information from multiple moving stimuli and showed that whereas increasing the number of stimuli improved speed discrimination, increasing the area of a single stimulus by the same factor did not⁶ (Fig. 1). These data do not show the classic summation with increasing area that has been reported for grating detection at threshold contrast⁷, and for the detection and direction discrimination of the motion of random dots at suprathreshold contrast^{8–10}. Thus, the data shown in Fig. 1 appear to be at odds with the view that speed discrimination is determined early, by local speed-tuned mechanisms^{2–5}. Such a framework predicts an improvement in discrimination with increasing stimulus size, either due to an increase in the stimulated area within a unit, or in the number of stimulated units.

To explain this surprising lack of summation, we propose that local speed estimates are influenced by the parsing of the image into discrete entities before or concurrently with the combination of speed information across space. Parsing effectively results in a single independent speed estimate per entity. If the speed estimates from each patch are independent samples from a noisy distribution, then in the multiple-patch condition, averaging across patches improves the estimate of speed. However, in the large-patch condition, there is only a single sample whose precision does not change with the size of the patch, and consequently there is no improvement in speed discrimination.

To test this hypothesis, we measured speed-discrimination thresholds using two complementary approaches. In the fusion experiment, we merged multiple gratings until they became a single stimulus, whereas in the fission experiment, we divided a single large grating into multiple stimuli. Our hypothesis predicts that thresholds will increase as multiple gratings merge to one, regardless of how this is achieved. This is contrary to the prediction of early vision models that assume that the visual field is tiled with arrays of specialized detectors that act in parallel and are insensitive to how the image is parsed.

In the fusion experiment, we merged multiple patches in stages as depicted in Fig. 2. In the multiple-patch condition, three grating patches were maximally separated, and had random spatial phase. In the in-phase condition, the three patches were closer and their phase relationship was consistent with three windows on a full-field grating. In the banana condition, the three patches were fused to form a single banana-shaped patch. In the large-patch condition, the stimulus was a single patch three times the area of the patch used in the multiple-patch condition. Speed-discrimination thresholds for the six observers are plotted, normalized to the threshold in the multiple-patch condition. Five of six observers show thresholds that increase as the multiple gratings are fused into a single patch. Thresholds increase when the stimuli are closer and have consistent phase, increase further when they are fused into the banana configuration, and increase further still going from the banana configuration to the large patch.

In the fission experiment, we divided a single large patch and separated the parts as depicted in Fig. 3. In the occluded condition, we superimposed on the large patch a cross that was darker than the background, thus giving the appearance of a single patch occluded by a cross. In the divided condition, we superimposed the same cross but with a luminance equal to the background, thus perceptually dividing the patch into four quadrants. In the multiple-patch condition, four gratings were maximally separated. Speed-discrimination thresholds for the six observers are plotted, normalized to the threshold in the multiple-patch condition. Starting with the large-patch condition, thresholds are unchanged by the occluder for four of our six observers. This result is consistent with the view that the stimulus in the occluded condition is amodally completed¹¹ and therefore perceived as a single patch moving behind a cross. However, thresholds do decrease in

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FIG. 1 **a**, A single frame of the condition with 6 grating patches. **b**, A single frame of the condition in which a single patch was $6 \times$ the size of the patch in **a**. **c** Speed-discrimination thresholds versus total stimulus area. Thresholds decreased with increasing grating number (solid symbols), but were unchanged with increased grating area (open symbols). A trial consisted of two intervals, each with the same number of patches. One of the intervals, picked randomly, had all its gratings moving at the reference speed ($5.3 \pm 0.7 \text{ deg s}^{-1}$) and the other had all its gratings moving at a faster speed (picked from 3 up-1 down staircase). Observers were asked to choose the interval with the faster patches. All gratings had a spatial frequency of 1.5 c deg^{-1} . The number of gratings, n , was kept fixed within a block of trials and was set to be 1, 2, 4 or 6, across blocks. The regular patches in **a** were windowed by a gaussian with a space constant (σ) of 0.4° . Patches whose area was n times larger had a σ that was a factor of $\sqrt{n}$ larger. The gratings were presented at a fixed eccentricity of 4° , and their angular separation in this experiment was $360^\circ/n$. The grating contrast was 20% and the duration of each interval was brief (195 ms) to minimize eye movements. For each experimental condition, thresholds were obtained from a psychometric function of proportion correct versus speed difference between the two intervals. The raw data were fitted with a Weibull function and thresholds estimated as the speed difference that gave 82% correct performance. The thresholds in **c** are the average of 4 observers' thresholds for that condition, normalized to the threshold for a single regular grating⁶.

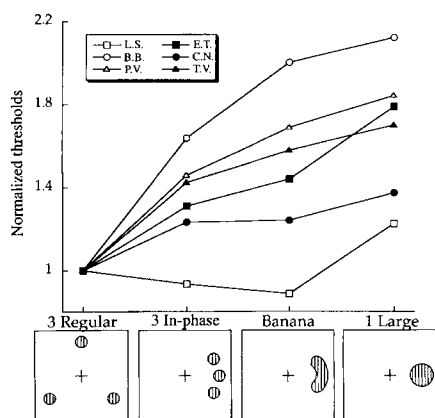
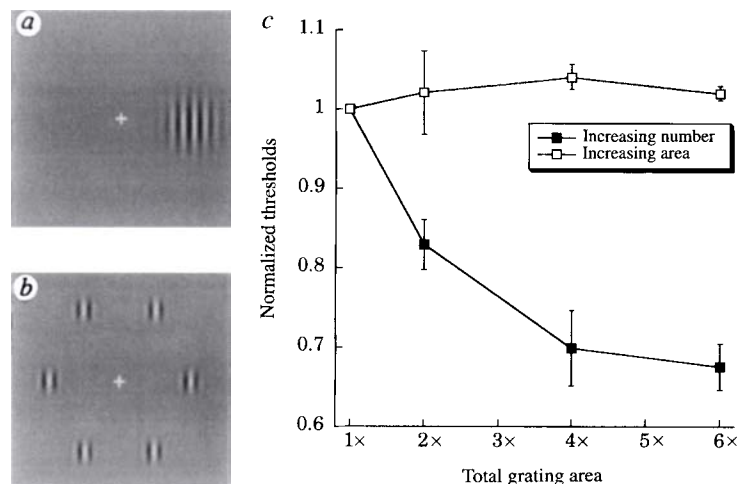


FIG. 2 Fusion experiment. Thresholds for six observers, normalized to that in the multiple-patch condition, are plotted versus spacing condition. The standard deviation for each observer is: L.S. (0.13), B.B. (0.15), P.V. (0.12), E.T. (0.26), C.N. (0.12) and T.V. (0.11). The latter 3 observers (represented by filled symbols here and in Fig. 3) were naive as to the purpose of the experiment. For 5 of 6 observers, thresholds increased as the multiple patches were fused into a single patch. The four conditions were: three regular grating patches maximally separated (the angular separation of their centres was 120°), three grating patches separated by 30° and with a phase relationship consistent with gaussian windows on a full-field grating, three patches fused into a single banana-shaped patch, and a single large circular grating with $3 \times$ the area of a regular patch. The spatial spread (σ) of the gaussian window for the regular patches in the first two conditions was 0.4° , and for the large patch in the last condition was 0.7° . The banana stimulus represented a fusion of three regular patches and therefore had the same width and gaussian profile; its (arc) length was $\sim 6^\circ$. The stimuli in the first three conditions had the same total bounding contour length. Each randomly appeared in one of four configurations on a given trial—that shown, and others rotated by 90° , 180° or 270° . Paired one-tailed *t*-tests across observers on the increase in threshold from one condition to the next were significant as follows: 3 regular to 3 in-phase ($P < 0.001$), 3 in-phase to banana ($P < 0.035$), and banana to large patch ($P < 0.005$). One-tailed tests are appropriate given our *a priori* hypothesis of a threshold increase.

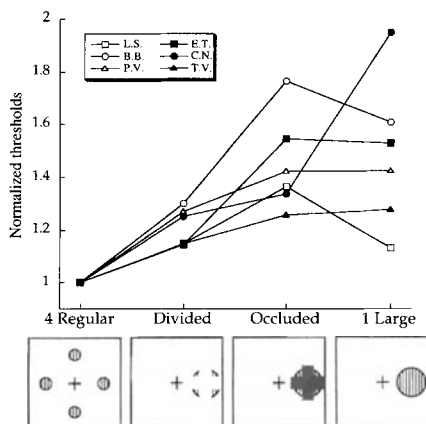


FIG. 3 Fission experiment. Thresholds for six observers, normalized to that in the multiple-patch condition, are plotted versus spacing condition. On average, the standard deviation of the threshold estimate for each observer is: L.S. (0.12), B.B. (0.13), P.V. (0.11), E.T. (0.13), C.N. (0.15) and T.V. (0.07). The four conditions were: a single large patch, the same patch with a superimposed dark cross, the same patch with a superimposed cross equiluminant with the background, and four patches, maximally separated. The last three conditions had the same area and peak contrast. The crosses had a nominal width of 1.3° . They had a centre portion 0.9° wide of constant contrast (10 and 0% in the occluded and divided conditions, respectively), with gaussian edges ($\sigma = 0.2^\circ$) tapering to mean luminance. Paired one-tailed *t*-tests across observers on the increase in threshold from one condition to the next were significant as follows: 4 regular to divided ($P < 0.005$), divided to occluded ($P < 0.008$). The thresholds for the occluded and single large patch conditions were not significantly different ($P = 0.38$).

the divided-patch case, although the only change from the occluded condition is the contrast of the superimposed cross. This is consistent with the divided-patch stimulus being perceived as four discrete parts that are not amodally completed, as in Bregman's compelling demonstration with the broken Bs (ref. 12). Thresholds decrease further in the multiple-patch case when the patches are maximally separated, indicating that proximity is also a factor.

These results show that image parsing affects the integration of local speed signals across space. The most dramatic demonstration of this effect is the significant increase in thresholds (19.7%) from the divided to the occluded condition, despite identical moving regions in both stimuli. A control experiment examined whether this increase was due to an increase in cross contrast *per se*. Simply increasing cross contrast from 10 to 30% caused no significant change in thresholds ($P = 0.41$, $n = 4$). For the four observers tested in both experiments, going from the divided to the occluded condition caused an average threshold increase 3.9 times larger than that seen in the increased-contrast control. The increase in thresholds from the divided to the occluded condition, and from the in-phase to the banana condition, in conjunction with our earlier results⁶, suggests that speed is estimated by pooling speed samples and that effectively only one independent sample is available from each image region that has been parsed as a distinct entity.

It has been shown that the detection of the trajectory of a moving dot embedded in noise can be disrupted if the trajectory is periodically broken by brownian motion, and is not restored by segregating the intervening brownian motion by colour or depth¹³. Although trajectory detection is sufficiently different from speed discrimination to make a direct comparison difficult, one interpretation of this result that is consistent with ours is that the brownian motion in the occluder does not affect local motion estimates in the non-occluded regions, but rather interferes with factors that would otherwise link parts of the motion trajectory together.

Several factors that potentially explain our results can be ruled out. Bounding contour length has little effect as thresholds remain largely unaffected by increasing the circumference of a patch (Fig. 1c) or by superimposing an occluder (Fig. 2). Furthermore, we have shown previously⁶ that eye movements, uncertainty about stimulus location, and variation in perceived speed with eccentricity¹⁴, are unlikely to cause the high thresholds in the case of a single patch. However, proximity and phase do appear to influence speed discrimination: bringing the patches closer and making their phase relationships consistent with a single grating cause thresholds to increase (Fig. 2). The grouping of different parts of the image into a single entity appears to make multiple local speed estimates inaccessible, in much the same way that observers are unable to access component speed when viewing coherently moving plaids^{15,16}. How might this occur? If the responses of speed-tuned units to multiple patches become increasingly correlated as the patches are brought closer or fused, the benefit of pooling speed information from multiple patches would be reduced^{17,18}. Alternatively, inhibitory pooling from surrounding units, either by subtractive^{19–21} or divisive^{22,23} mechanisms, could decrease the response to extended stimuli, lowering the signal-to-noise ratio and thus increasing thresholds.

The fact that the thresholds in our experiment remain unchanged in the presence of partial occlusion is consistent with the results of He and Nakayama^{24,25}, who advocate an early parsing of the image into surfaces. Our results extend this view by showing that the parsing of multiple patches, even on a single surface, affects speed perception. Furthermore, there is mounting physiological evidence that neurons in early visual processing areas such as V1 (ref. 26) and MT (ref. 27) are sensitive to image segmentation cues located outside their classical receptive field. Our data provide complementary psychophysical evidence that has important implications for the interaction between local neural mechanisms that code speed. The effect of both proximity and segmentation on speed discrimination suggest that local speed

mechanisms do not act in isolation, but rather in assemblies that have both neighbouring and long-range interactions. ■

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1. De Valois, R. L. & De Valois, K. K. *Spatial Vision* (Oxford University Press, New York, 1990).
2. Maunsell, J. H. R. & Van Essen, D. C. *J. Neurophys.* **49**, 1127–1147 (1983).
3. McKee, S. P., Silverman, G. H. & Nakayama, K. *Vision Res.* **26**, 609–618 (1986).
4. Movshon, J. A., Newsome, W. T., Gizzi, M. S. & Levitt, J. B. *Invest. ophthalm. Vis. Sci. (suppl.)* **29**, 327 (1988).
5. Pasternak, T. & Merigan, W. H. *Cerebral Cortex* **4**, 247–259 (1994).
6. Verghese, P. & Stone, L. S. *Vision Res.* **35**, 2811–2823 (1995).
7. Robson, J. G. & Graham, N. *Vision Res.* **21**, 409–418 (1981).
8. Downing, C. J. & Movshon, J. A. *Invest. ophthalm. Vis. Sci. (suppl.)* **30**, 72 (1989).
9. Watamaniuk, S. N. J. & Sekuler, R. *Vision Res.* **32**, 2341–2347 (1992).
10. Morrone, M. C., Burr, D. C. & Vaina, L. M. *Nature* **376**, 507–509 (1995).
11. Kanizsa, G. *Organization in Vision: Essays on Gestalt Perception* (Praeger, New York, 1979).
12. Bregman, A. L. in *Perceptual Organization* (eds Kubovy, M. & Pomerantz, J. R.) (Erlbaum, Hillsdale, NJ, 1981).
13. Watamaniuk, S. N. J. & McKee, S. P. *Nature* **377**, 729–730 (1995).
14. Johnston, A. & Wright, M. J. *Vision Res.* **26**, 1099–1109 (1986).
15. Welch, L. *Nature* **337**, 734–736 (1989).
16. Welch, L. & Bowne, S. F. *Perception* **19**, 425–435 (1990).
17. Britten, K. H., Shadlen, M. N., Newsome, W. T. & Movshon, J. A. *J. Neurosci.* **12**, 4745–4765 (1992).
18. Gray, C. M., Konig, P., Engel, A. K. & Singer, W. *Nature* **338**, 334–337 (1989).
19. Allman, J., Miezin, F. & McGuinness, E. *Perception* **14**, 105–126 (1985).
20. Born, R. T. & Tootell, R. B. H. *Nature* **357**, 497–499 (1992).
21. Watson, A. B. & Eckert, M. P. *J. opt. Soc. Am. A* **11**, 496–505 (1994).
22. Legge, G. E. & Foley, J. M. *J. opt. Soc. Am. A* **70**, 1459–1470 (1980).
23. Heeger, D. J. *Visual Neurosci.* **9**, 181–197 (1992).
24. He, Z. J. & Nakayama, K. *Nature* **359**, 231–233 (1992).
25. He, Z. J. & Nakayama, K. *Nature* **367**, 173–175 (1994).
26. Zipser, K. *Invest. ophthalm. Vis. Sci. (suppl.)* **34**, 3171 (1994).
27. Albright, T. D. & Stoner, G. R. *Proc. natn. Acad. Sci. U.S.A.* **92**, 2433–2440 (1995).

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Bidirectional modification of CA1 synapses in the adult hippocampus *in vivo*

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MEMORIES are believed to be stored by synaptic modifications. One type of activity-dependent synaptic modification, long-term potentiation (LTP), has received considerable attention as a possible memory mechanism, particularly in hippocampus¹. However, use-dependent decreases in synaptic strength can store information as well. A form of homosynaptic long-term depression (LTD) has been described and widely studied in the CA1 region of the developing hippocampus *in vitro*^{2–4}. However, the relevance of this model of LTD to memory has been questioned because of failures to replicate it in the adult brain *in vitro*⁵ and, more recently, *in vivo*⁶. Here we re-examine this important issue and find that homosynaptic LTD can in fact be elicited in the adult hippocampus *in vivo*, that it has all the properties described in immature CA1 *in vitro*, and that LTD and LTP are reversible modifications of the same Schaffer collateral synapses. Thus homosynaptic LTD is not peculiar to brain slices, nor is it only of developmental significance. Rather, our data suggest that the mechanisms of LTP and LTD may be equal partners in the mnemonic operations of hippocampal neural networks.

We emulated *in vivo* the precise stimulation and recording configuration that has been used successfully to elicit homosynaptic LTD *in vitro* (Fig. 1a). Low-frequency stimulation (LFS; 900 pulses at 1–3 Hz) of the ipsilateral Schaffer collaterals resulted in

a significant ($P < 0.01$) and stable depression of the evoked extracellular excitatory postsynaptic potential (EPSP) (Fig. 1b, c). The initial slope of the field EPSP 30 min after LFS was $84.4 \pm 5.3\%$ (mean $\pm$ s.e.m.) of the average baseline value for 1 Hz stimulation ($n = 9$) and $82.9 \pm 3.5\%$ for 3 Hz stimulation ($n = 4$).

To assess whether the LTD was restricted only to the synapses that received LFS, we monitored responses to stimulation of a second pathway originating in the contralateral CA1. We found that LFS of the ipsilateral Schaffer collaterals produced LTD only of the responses to stimulation of this input (Fig. 1c). Thus, just as in immature hippocampal slices, LFS of the Schaffer collaterals can produce homosynaptic (input-specific) LTD *in vivo*. Figure 1c also shows that there is a limit to the magnitude of LTD produced by repeated episodes of LFS.

We assessed the dependence of LTD on stimulation frequency *in vivo* by applying 900 pulses at 10 Hz, a frequency that slice studies suggest produces little net long-term synaptic modification². Our data confirm that 10 Hz stimulation produces no significant change in synaptic response *in vivo*, even if it is delivered following induction of LTP (Fig. 1d).

High-frequency stimulation (HFS) after establishment of LTD produced LTP (Fig. 2a). However, because the responses are population EPSPs, this finding alone cannot support the conclusion that depressed synapses can subsequently be potentiated. To address this important issue, we first saturated LTD by applying three 1 Hz trains to the Schaffer collaterals (EPSP slope = $67.6 \pm 4.5\%$ of initial baseline; $n = 3$), and then we produced potentiation relative to this depressed baseline by applying HFS (Fig. 2b). Subsequent application of LFS returned the response to the LTD saturation level ($65.1 \pm 7.7\%$ of initial baseline). The simplest interpretation for the observation that

HFS unsaturates LTD is that depressed synapses can be potentiated.

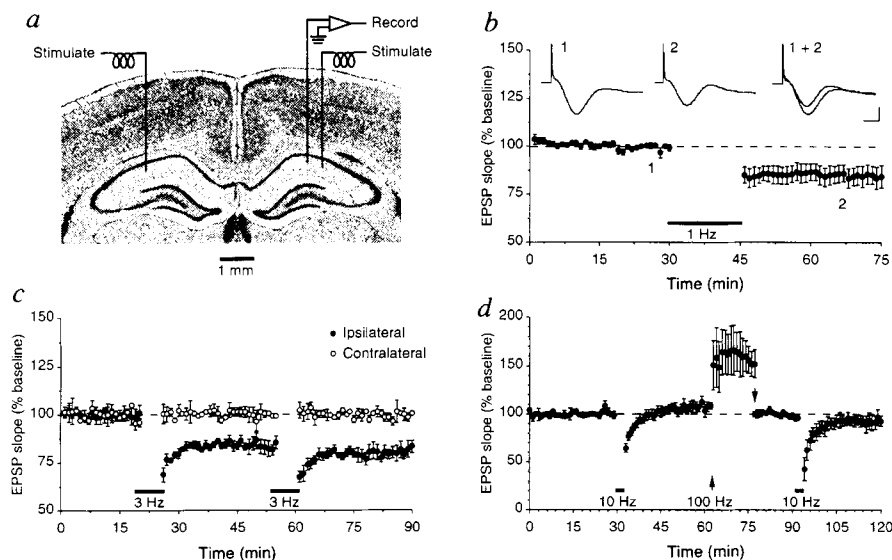
We also did the complementary experiment in which LTP was first saturated by applying repeated episodes of HFS (EPSP slope = $135.7 \pm 10.8\%$ of baseline; $n = 3$), followed by induction of LTD relative to this potentiated baseline (Fig. 2c,d). Subsequent application of HFS restored the response to the LTP saturation level ($130 \pm 11.6\%$ of initial baseline). Taken together, these findings strongly suggest that Schaffer collateral synapses in adult CA1 are bidirectionally modifiable *in vivo*.

The characteristics of the homosynaptic LTD we have observed in adult CA1 *in vivo* are identical to those described in a number of slice studies²⁻⁴, suggesting that they are explained by common mechanisms. The mechanism for LFS-induced LTD *in vitro* involves NMDA (*N*-methyl *D*-aspartate) receptors. Therefore, we did experiments to determine whether LTD *in vivo* is sensitive to NMDA receptor blockade (Fig. 3). Application of 1 Hz conditioning stimulation following administration of the competitive NMDA-receptor antagonist CPP (($\pm$)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; 10 mg kg^{-1} injected i.p.) failed to produce LTD ($100.7 \pm 2.3\%$ of baseline; $n = 4$). As expected, CPP-treated animals also failed to show LTP after HFS⁷. Thus, bidirectional modifications of synapses using low- and high-frequency stimulation in CA1 require activation of NMDA receptors.

LTP has attracted interest as a memory mechanism partly because of its longevity. To investigate the duration of homosynaptic LTD, in several experiments we monitored responses for $> 5 \text{ h}$ after a single episode of LFS (1 Hz, 900 pulses). In all cases, LTD remained stable for as long as the preparation was viable. An example of stable, long-lasting homosynaptic LTD is shown in Fig. 4a. Thus LTD, like LTP, can store information long enough to contribute to a hippocampal memory store.

FIG. 1 Induction of homosynaptic LTD using low-frequency conditioning stimulation. a, Recording and stimulating electrode configuration in dorsal hippocampus. b, 900 pulses delivered at 1 Hz results in LTD of the EPSP slope ($n = 9$). Field potentials (average of 10 consecutive sweeps) are from one case at times indicated by numerals (scale bars: 5 ms, 2.5 mV). c, LFS-induced LTD is input-specific and saturable ($n = 5$). d, Homosynaptic LTD is frequency dependent. 900 pulses delivered at 10 Hz produces no lasting effect on EPSP slope, irrespective of whether conditioning stimulation is applied 'de novo' or following induction of LTP by high-frequency stimulation (indicated by upward arrow; $n = 5$). For ease of comparison, data have been renormalized after LTP induction (indicated by downward arrow).

METHODS. Adult, male Sprague-Dawley rats (250–500 g) were anaesthetized with sodium pentobarbital (65 mg kg^{-1} , i.p.), placed in a stereotaxic frame, and maintained at $37 \pm 0.5^\circ \text{C}$. A monopolar recording electrode was positioned in the CA1 stratum radiatum region of the hippocampus of one hemisphere (coordinates in mm from bregma and the midline: 3.5 posterior; 2.3–2.5 lateral). A monopolar stimulating electrode was positioned $\sim 0.2 \text{ mm}$ lateral to the recording electrode in order to directly activate the ipsilateral Schaffer collaterals. In some animals a second monopolar stimulating electrode was positioned in a homotopic site in the contralateral hippocampus. All electrodes were constructed from Teflon-insulated stainless-steel wire (75 μm) cut flat at the tip. Final depths of recording and stimulating electrodes were adjusted to optimize the magnitude of the evoked response. Screws inserted into the skull overlying the cerebellum served as recording ground and stimulus anode. Field EPSPs were elicited using stimuli of 0.2 ms duration. Evoked responses were amplified and filtered at 0.1 and 3.0 kHz (1/2 amplitude), digitized at 20 kHz and stored on a computer for later analysis. The initial slope of the field EPSP was used as a measure of the magnitude of the response. At the beginning of each



experiment a full input-output curve was generated, and a stimulus strength eliciting an EPSP slope of 50–65% of maximum was used for the remainder of experimentation (30–65 μA). Baseline measurements were collected using single stimuli applied every 15–30 s. LTD was induced using LFS, consisting of 900 pulses delivered at 1 or 3 Hz. To induce LTP, two 1-s bursts of 100 Hz separated by 10 s were applied. At the end of experimentation, rats were killed, their brains removed and sectioned and stained with thionin to verify recording and stimulating electrode placements. Data are expressed as a percentage of the mean response magnitude recorded during the baseline period. Paired or independent two-tailed *t*-tests were used to assess statistical significance. In a small number of cases ($< 10\%$), LFS failed to produce LTD. In these same animals, HFS was also ineffective in producing LTP; these cases were therefore excluded from analysis.

Previous attempts to induce LTD in adult CA1 *in vivo* using LFS have failed^{6,8}. In these studies, synaptic responses were evoked in CA1 by stimulating the commissural inputs instead of the ipsilateral Schaffer collaterals. To investigate whether this procedural difference underlies the discrepancy between laboratories, in an additional series of experiments we examined the effects of LFS of the contralateral input. We confirmed that 1 Hz stimulation produces little LTD of the commissural input ($96.2 \pm 1.4\%$ of baseline, $n = 4$; Fig. 4b). High-frequency stimulation, however, was still able to produce LTP in these synapses.

Studies of Schaffer collateral LTD in hippocampal slices prepared from adult animals have yielded conflicting results (compare refs 2 and 5). The finding that LTD can be induced in the brain *in situ* is therefore of considerable importance as it demonstrates that LTD is not peculiar to particular conditions *in*

vitro. We believe our success in eliciting LTD in Schaffer collateral synapses, both *in vivo* and *in vitro*, is attributable to the proximity of the stimulation and recording electrodes and the use of a restricted range of stimulation intensities⁹.

Many studies *in vitro* suggest that homosynaptic LTD results from a critical level of NMDA receptor activation and postsynaptic Ca^{2+} entry⁴. Therefore, it is reasonable to expect that types of stimulation other than the LFS used here may satisfy the conditions for homosynaptic LTD induction. Indeed, repetitive

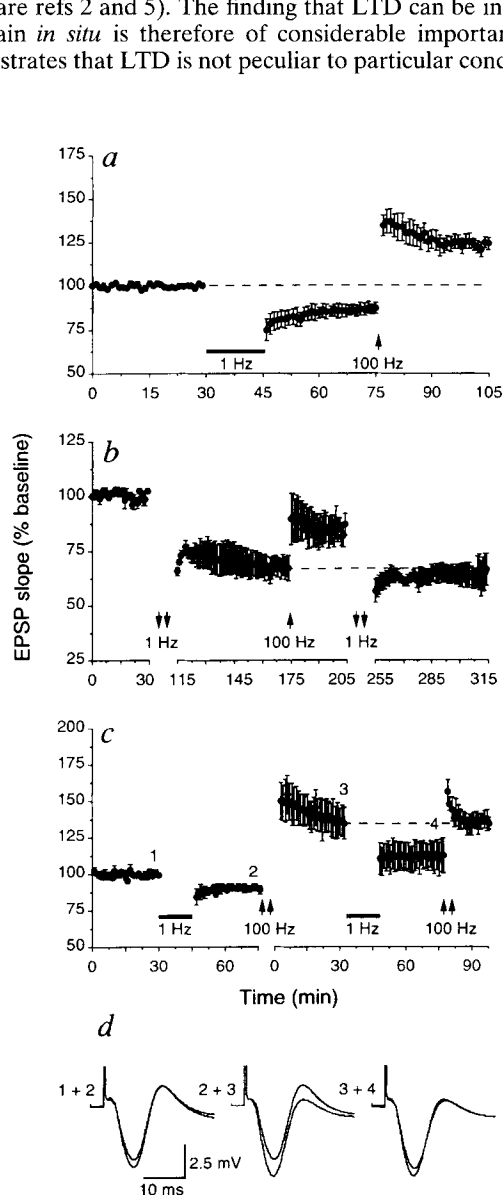


FIG. 2 Bidirectional plasticity in CA1 *in vivo*. *a*, Homosynaptic LTD induced by LFS (EPSP slope = $87.1 \pm 3.4\%$ of baseline) can be fully reversed and replaced with LTP ($123.3 \pm 3.8\%$ of initial baseline) following application of HFS (indicated by upward arrow; $n = 5$). *b*, LTD can be unsaturated by HFS. LTD was saturated by application of three 900-pulse trains of 1 Hz stimulation (downward arrows). The response could be potentiated by HFS (upward arrow) and returned to the LTD saturation level by subsequent application of LFS ($n = 3$). *c*, LTP can be unsaturated by LFS. Following LTD induction, LTP was saturated by application of HFS (3–6 1-sec trains of 100 Hz). The response could then be depressed by application of 900 pulses at 1 Hz and returned to the LTP saturation level by subsequent applications of HFS (2–4 trains, $n = 3$). *d*, Field potentials (average of 10 consecutive sweeps) from one case at times indicated by numerals in *c*.

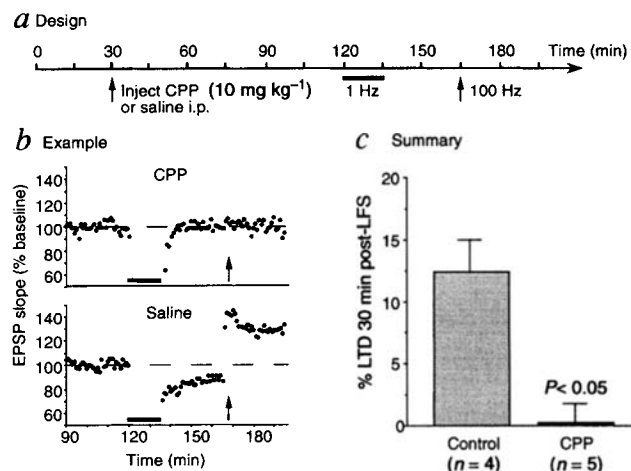


FIG. 3 The NMDA receptor antagonist CPP blocks homosynaptic LTD induction. *a*, Time line depicting experimental design. Animals received CPP (10 mg kg^{-1} i.p.) or an equal volume of saline following 30 min of baseline recording. 90 min following drug administration, 1 Hz conditioning stimulation was applied, followed 30 min later by HFS. *b*, Examples from one animal receiving CPP and a saline control animal. Note the lack of both LTD and LTP in animal receiving CPP. *c*, Data summary.

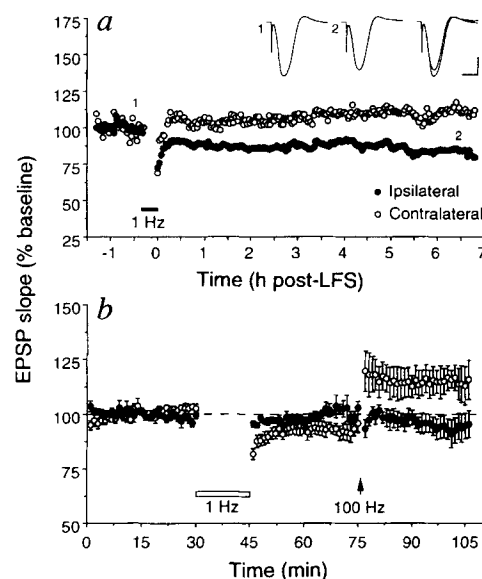


FIG. 4 *a*, Homosynaptic LTD induced by a single episode of LFS (1 Hz, 900 pulses) is stable for as long as the preparation is viable. In this example, LFS of the ipsilateral Schaffer collaterals produced LTD that was stable for > 6.5 h. Displayed field potentials were evoked by stimulation of the ipsilateral Schaffer collaterals (averages of 10 consecutive sweeps) at times indicated by numerals. Scale bars: 2 mV, 10 ms. *b*, Responses evoked by contralateral stimulation of CA1 support little LTD after LFS, but show robust LTP after 100 Hz stimulation ($n = 4$).

paired-pulse stimulation of the commissural input reportedly can produce LTD in CA1 *in vivo*⁸. Although issues of input specificity and bidirectional plasticity were not directly addressed in that study, the LTD was sensitive to NMDA receptor blockade. Thus, commissural LTD and Schaffer collateral LTD may require different types of stimulation patterns, but use the same mechanisms.

Our findings show that homosynaptic LTD is not peculiar to the brain slice preparation, and that synapses in adult CA1 support long-term, bidirectional modifications. Demonstration of bidirectional synaptic plasticity in the adult hippocampus has significant impact on hypotheses concerning how hippocampal neural networks store information. Many CA1 neurons are selective for positions in space, and this selectivity shifts as animals learn new spatial environments^{10,11}. Shifts in selectivity require that neurons acquire responsiveness to new stimuli and lose responsiveness to previously effective stimuli¹². The mechanisms of homosynaptic

LTP and LTD, functioning together, are ideally suited to account for this type of receptive field plasticity. □

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1. Bliss, T. V. P. & Collingridge, G. L. *Nature* **361**, 31–39 (1993).
2. Dudek, S. M. & Bear, M. F. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4363–4367 (1992).
3. Mulkey, R. M. & Malenka, R. C. *Neuron* **9**, 967–975 (1992).
4. Bear, M. F. & Malenka, R. C. *Curr. Opin. Neurobiol.* **4**, 389–399 (1994).
5. Bashir, Z. I. & Collingridge, G. L. *Expl. Brain Res.* **100**, 437–43 (1994).
6. Errington, M. L. et al. *J. Neurophysiol.* **74**, 1793–1799 (1995).
7. Abraham, W. & Mason, S. *Brain Res.* **462**, 40–46 (1988).
8. Thiels, E., Barrionuevo, G. & Berger, T. W. *J. Neurophysiol.* **71**, 3009–3016 (1994).
9. Kerr, D. S. & Abraham, W. C. *Proc. natn. Acad. Sci. U.S.A.* **92**, 11637–11641 (1995).
10. Breese, C., Hampson, R. & Deadwyler, S. J. *Neurosci.* **9**, 1097–1111 (1989).
11. Wilson, M. & McNaughton, B. *Science* **261**, 1055–1058 (1993).
12. Bienenstock, E. L., Cooper, L.N. & Munro, P. W. J. *Neurosci.* **2**, 32–48 (1982).

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A role for the proteasome regulator PA28 α in antigen presentation

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CYTOTOXIC T cells recognize viral proteins as peptide fragments which are produced in the cytosol and transported on major histocompatibility complex (MHC) class I proteins to the cell surface¹. Viral peptides that meet the stringent binding characteristics of class I proteins are generated by the 20S proteasome^{2,3}. The interferon (IFN)- γ -inducible activator of the 20S proteasome, PA28 (refs 4–6), strongly influences the proteasomal cleavage pattern *in vitro*⁷. This led us to investigate whether changes in cellular levels of PA28 affect the efficiency of viral antigen processing. A mouse fibroblast line expressing the murine cytomegalovirus pp89 protein was transfected with either the human or murine gene encoding the PA28 α subunit, which is sufficient to activate the peptide-hydrolysing activity of the 20S proteasome *in vitro*. Here we report that enhanced expression of PA28 α at a level similar to that obtained after IFN- γ induction resulted in a marked enhancement of recognition by pp89-specific cytotoxic T cells; the presentation of influenza nucleoprotein was also significantly improved. These results demonstrate a fundamental *in vivo* function for PA28 α in antigen processing.

Two subunits, PA28 α and PA28 β , with an apparent relative molecular mass of about 29,000 (M_r 29K) are found in human PA28 activator complexes of the 20S proteasome^{8,9}. They form ringlike hexa- or heptameric complexes of $M_r \sim 200K$ that bind reversibly to the cylindrical 20S proteasome and activate its peptide-hydrolysing activity¹⁰. The subunit stoichiometry of PA28 complexes has not yet been determined. The primary structures of the PA28 α and β subunits have 47% identity, and both are inducible by IFN- γ to a comparable extent^{11,12}. Only

PA28 α contains KEKE motifs, which are putatively involved in the interaction between proteasome and PA28 (ref. 13). Complexes of recombinant PA28 α protein display biochemical properties and peptide-hydrolysing activity very similar to native, human erythrocyte PA28 complexes⁸, so we constitutively overexpressed PA28 α in the mouse fibroblast clone B8, which expresses the pp89 immediate early gene product of murine cytomegalovirus (MCMV). The efficiency of antigen processing was monitored by cytotoxic T cells specific for the pp89-derived peptide YPHFMPNTL, presented by the class I allele H-2L^d (ref. 14). Enhanced expression of the human PA28 α protein in B8 cells resulted in a markedly enhanced recognition by pp89 peptide-specific cytotoxic T cells. To achieve the same lysis in PA28 α transfectants, as compared to B8 wild-type cells, about 20-fold fewer cytotoxic T cells were required. A representative result obtained with B8 wild-type cells and human PA28 α transfectants, designated BP α h.1 and BP α h.5, respectively, is shown in Fig. 1a. To verify this finding in a homologous system, we cloned the murine PA28 α complementary DNA (A.S., unpublished data) for overexpression in B8 cells. Enhanced expression of the murine protein, which is 94% identical to its human homologue, also led to a similar increase in susceptibility to pp89-specific T cell-mediated lysis (see clones BP α m.4 and BP α m.5 in Fig. 1a). This increase in specific lysis was due to the PA28 α overexpression only, because transfection of the puromycin-resistance gene alone, or together with LMP2 and LMP7 proteins expressed from the same expression vector, had no effect in cytotoxicity assays (Fig. 1b). The deficiency in pp89 antigen presentation of B8 wild-type cells was due to a lack of pp89 nonamer peptide, as exogenous peptide enhanced the recognition of B8 cells to levels obtained with PA28 α transfectants (Fig. 1c).

To quantify the expression of human and murine PA28 α in the recipient clone B8 and in transfectants, quantitative western-blot analysis was performed. Total lysates were blotted and probed with a PA28 α -specific polyclonal antibody, and subsequent stripping of the blots were reprobed with an antibody specific for the constitutive proteasome subunit C3. Densitometric scans of the blots shown in Fig. 2a yielded expression of PA28 α in all clones tested that was threefold higher than in B8 cells after normalization on total protein or C3 expression. PA28 α expression in the transfectants was found to be comparable with that following stimulation of B8 cells with IFN- γ for 72 h (Fig. 2b). To exclude an effect of PA28 α transfection on pp89 expression, we quantified the expression of pp89 protein in B8 cells and PA28 α transfectants. Immunoprecipitation of pp89 from lysates of metabolically labelled cells demonstrated that the amount of pp89 expression was identical in B8 cells and its PA28 α transfectants (Fig. 2c). The PA28 α -mediated increase in antigen presentation was not due to a higher pp89 turnover, as no obvious enhancement of pp89 degradation was found for PA28 α transfectants in pulse-chase experi-

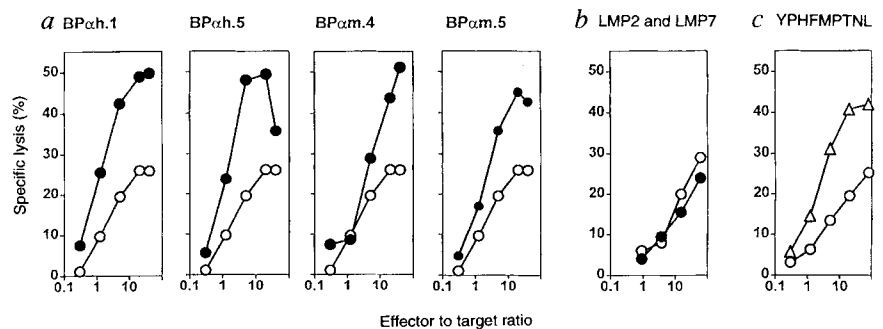
ments (Fig. 2d). Hence the effect of PA28 α must be ascribed to a higher efficiency of antigen processing from degraded proteins.

The stimulation of B8 cells with IFN- γ for 3 days increases cell-surface expression of MHC class I molecules by about tenfold (M.G., unpublished observation). To test whether an enhanced PA28 α expression would suffice to upregulate MHC expression we compared the cell-surface expression of H-2D^d, H-2L^d and H-2K^d class I molecules of B8 cells and PA28 α transfectants by flow-cytometric analysis (Fig. 3). None of the transfectants showed a significantly altered MHC class I surface expression when compared to B8 cells. Thus the marked increase in antigen presentation of pp89 in PA28 α transfectants as compared to B8 cells

cannot be attributed to a general increase in MHC class I surface expression.

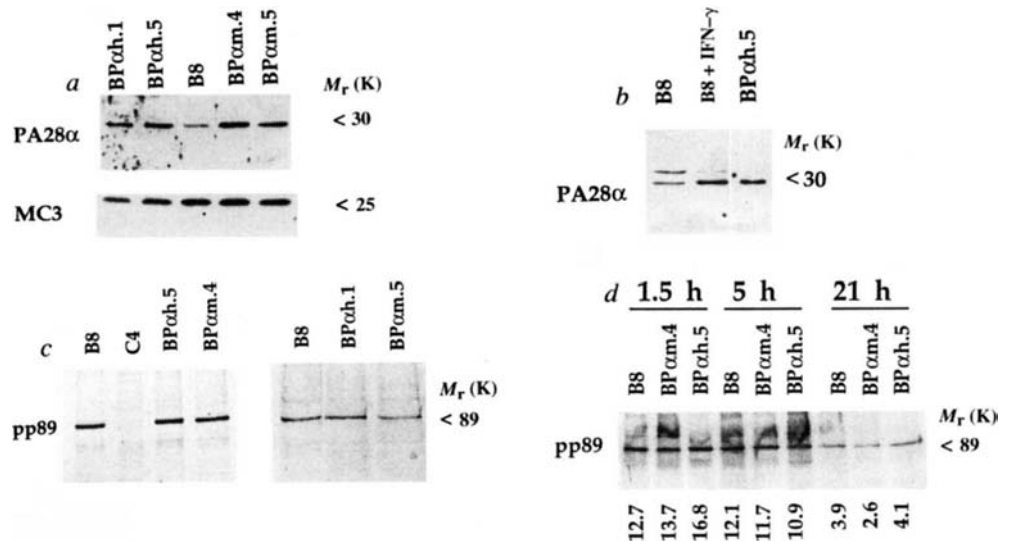
To investigate whether the PA28 α -dependent improvement in antigen presentation was confined to the MCMV pp89 antigen, we examined the presentation of another viral antigen. B8 cells and the PA28 α transfectant BP α m.5 were infected with the influenza virus strain A/PR/8/34/4, and the presentation of an influenza nucleoprotein-derived peptide on H-2K^d class I molecules was examined (Fig. 4a, c). The enhanced PA28 α expression in the transfectant resulted in an improvement in influenza nucleoprotein presentation to a similar extent as was found for the MCMV pp89 protein. The increase in cytotoxicity with alloreactive T

FIG. 1 a, Analysis of antigen presentation of endogenously expressed MCMV pp89 protein in B8 cells (open circles) and its transfectants (filled circles) BP α h.1, BP α h.5 (human PA28 α) and BP α m.4, BP α m.5 (murine PA28 α) in cytolytic assays. b, Overexpression of LMP2 and LMP7 in B8 cells (B8, open circles; LMP2/LMP7 double transfectant, filled circles) using the same expression and puromycin-resistance constructs⁷ did not affect pp89 antigen presentation. c, Exogenous administration of 10⁻⁸ M synthetic pp89 nonamer peptide (YPHFMTNL, open triangles) elevates B8 cytotoxicity (open circles) to levels obtained in PA28 α transfectants. The effector T cells¹⁶ were specific for the immunodominant pp89 nonamer YPHFMTNL presented on H-2L^d. Data represent the means of three replicate cultures; the results shown were verified in three independent experiments. METHODS. Plasmid pPA α h was generated by cloning the human PA28 α cDNA into the pCR3 eukaryotic expression vector using the TA Cloning Kit (Invitrogen, San Diego). The insert was obtained by polymerase chain reaction (PCR) using primers GTGGGATCCATGGCCATGCTCAGGCTCCAGCC and GTGAGATCTTCAATAGATCATTCCTTTG, and the PA28 α plasmid pLGUP.I-5111 as template¹¹; no PCR errors were detectable in the construct. The mouse PA28 α cDNA was cloned from a λ gt11 liver cDNA library



(Clontech, Palo Alto, CA), according to the manufacturers' protocol using the human cDNA as a probe. The full-length cDNA was sequenced and cloned into the pSG5 expression vector (Stratagene) using EcoRI restriction sites (pPA α m). B8 cells were stably transfected with 8 μ g pPA α h or pPA α m and 2 μ g puromycin-resistance plasmid pLXSP by calcium phosphate precipitation. Target cells for cytotoxic assays were labelled for 90 min with Na₂⁵¹CrO₄. A standard 4-h cytotoxic assay was performed with 1,000 target cells and graded numbers of effector cells in two- or four-fold dilution steps¹⁶.

FIG. 2 Quantification of PA28 α and pp89 protein expression in B8 cells and subclones transfected with human (BP α h.1, BP α h.5) and murine (BP α m.4, BP α m.5) PA28 α -expression constructs; comparison of PA28 α expression in B8 cells following IFN- γ induction and in transfectant clones. a, Top, expression levels of PA28 α in indicated clones was determined on a western blot by densitometric evaluation of ECL-exposed films. The overexpression factor compared to B8 was 2.8 for BP α h.1, 3.1 for BP α h.5, 2.9 for BP α m.4, and 2.7 for BP α m.5. The blots were stripped and reprobed with an antiserum detecting the constitutive proteasome subunit C3 (ref. 17) (bottom). Ponceau staining of total protein on the blots or C3 expression were used for the normalization of PA28 α expression and yielded identical results; quantifications were performed within the linear range of immunoblotting and densitometry. b, B8 cells were grown in culture for 3 days in the absence or presence of 20 U ml⁻¹ recombinant murine IFN- γ (Boehringer) and PA28 α expression was quantified as described above. PA28 α expression levels relative to B8 were 2.9 for B8 + IFN- γ and 3.0 for the PA28 α transfectant BP α h.5. c, Immunoprecipitation of MCMV pp89 from lysates of metabolically labelled cells. Clone B8 was obtained by transfection of the Balb/c-derived embryonic-fibroblast line C4 with pIE100 encoding MCMV pp89 (ref. 18); clones B8, BP α h.1, BP α m.4 and B8, BP α h.1, BP α m.5 (separate experiments) are described in Fig. 1. Radioactivity in the 89K band was quantified on a phosphorimager and found to be identical for B8 cells and all PA28 α transfectants. d, Pulse-chase experiment comparing the degradation of pp89 immunoprecipitated



from B8, BP α m.4 and BP α h.5 after 1 h of pulse and indicated periods of chase. Numbers below the lanes represent pixel values $\times 1,000$ in pp89 bands determined with a phosphorimager.

METHODS. Western blots of total lysates (quantified by absorbance at 280 nm) were performed with a PA28 α -reactive rabbit antiserum (L.K., unpublished data) according to standard protocols. For immunoprecipitation, 1×10^6 cells were labelled for 1 h (c, d) with ³⁵S-methionine. Lysis and immunoprecipitation from portions of 6×10^6 DPM with the monoclonal pp89-specific antibody 6-58.1 (ref. 19) were performed as described²⁰. Proteins were separated on 8% SDS-polyacrylamide gel electrophoresis, dried and visualized by autoradiography.

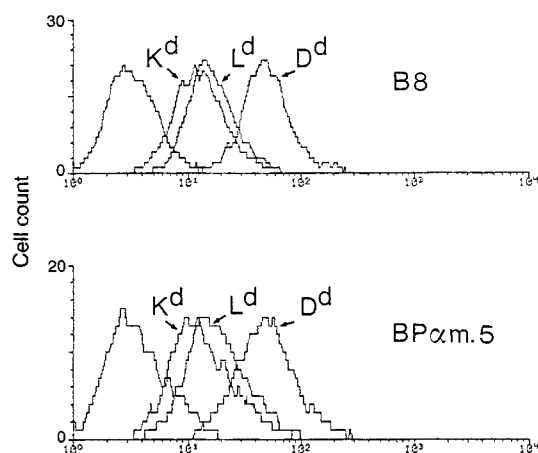
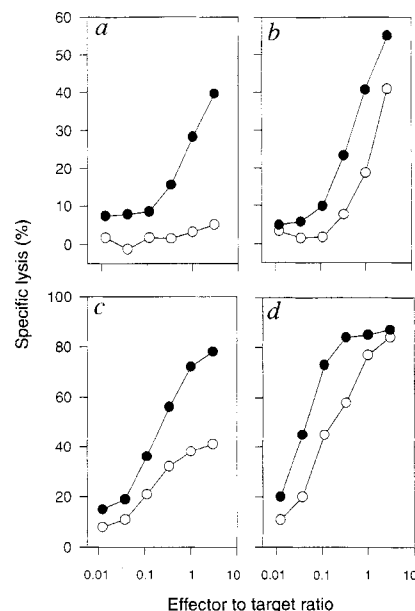


FIG. 3 Flow-cytometric analysis of MHC class I surface expression on B8 cells (top) and a representative PA28 α transfectant (BP α m.5) (bottom). No significant difference in surface expression of the murine class I proteins H-2D^b, H-2L^d, H-2K^d (as indicated) were detected between B8 wild type and any of the analysed human (12 clones) or murine (9 clones) PA28 α transfectants. Second-stage controls on the left are not assigned. METHODS. Adherent cells were recovered from culture dishes with calcium- and magnesium-free medium, washed and stained according to standard protocols with monoclonal antibodies 19/191 (anti H-2D^b), 28-14-8S (anti H-2L^d), and 15-5-5S (anti H-2K^d) and a sheep anti-mouse immunoglobulin-FITC conjugate (Silenius, Hawthorn, Australia) as second-stage reagent. The analysis was performed with a FACSCAN flow-cytometer and LYSIS II (Becton Dickinson, Heidelberg) software.

FIG. 4 Recognition of B8 cells (open circles) and the murine PA28 α transfectant BP α m.5 (filled circles) after infection with influenza A/PR/8/34/4 virus by: in *a* and *c*, the H-2K^d-restricted, influenza nucleoprotein-specific cytotoxic T lymphocyte (CTL) line HASI²¹; and in *b-d*, a C57Bl/6 anti BALB/c alloreactive CTL line. Two (*a*, *b* and *c*, *d*) of four independent experiments with similar results are shown. Target cells were infected with 500 HAU influenza virus A/PR/8/34/4, and cytotoxic assays were performed as described²¹. The recognition of uninfected B8 and BP α m.5 cells by HASI was < 5%, and the recognition of EL-4 cells by the alloreactive CTL line was < 4%. The alloreactive CTL line was generated by incubation of C57Bl/6 splenocytes with irradiated BALB/c spleen cells for 5 days, followed by weekly stimulations with irradiated BALB/c spleen cells as described¹⁵.



cells was only threefold (Fig. 4*b, d*), reflecting the heterogeneous specificity of alloreactive T cells, only some of which are peptide specific¹⁵. For both influenza nucleoprotein and MCMV pp89, overexpression of the IFN- γ -inducible proteasome subunits LMP2 and LMP7 in B8 cells either alone or together⁷ did not lead to enhanced antigen presentation, in striking contrast to PA28 α .

The demonstration that dominant T-cell epitopes of two viruses are presented more efficiently owing to enhanced expression of PA28 α , as well as similar effects with alloreactive T cells, establishes an *in vivo* function for PA28 in antigen processing. It also raises questions about the molecular mechanism by which PA28 mediates this effect. It is theoretically feasible that PA28 facilitates a transfer of antigenic peptides into the endoplasmic reticulum, or that it renders more efficient the generation of antigenic peptides by the 20S proteasome. Experimental results have suggested that PA28 exerts its effect on antigen presentation by altering the cleavage characteristics of the 20S proteasome⁷. Although in 20S proteasome end-point digests of pp89-derived 25-mer polypeptides, less immunodominant nonamer was recovered in the presence of PA28 (ref. 7), a recent analysis of products obtained after short incubation times demonstrated that about tenfold more nonamer and a potential precursor of the latter were recovered in the presence of PA28 (T.P.D., T. Ruppert, M.G., P.K., L.K., U.K., S. Stevanovic, H.S. and H.R., manuscript submitted). This result correlates with our *in vivo* findings, and is consistent with a proposed function for PA28 in antigen presentation. □

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- Rammensee, H. G., Falk, K. & Rötzschke, O. A. *Rev. Immun.* **11**, 213–244 (1993).
- Boes, B. et al. *J. exp. Med.* **179**, 901–909 (1994).
- Rock, K. L. et al. *Cell* **78**, 761–771 (1994).
- Yukawa, M. et al. *Biochem. biophys. Res. Commun.* **178**, 256–262 (1991).
- Chu-Ping, M., Slaughter, C. A. & DeMartino, G. N. *J. Biol. Chem.* **267**, 10515–10523 (1992).
- Dubiel, W., Pratt, G., Ferrell, K. & Rechsteiner, M. *J. Biol. Chem.* **267**, 22369–22377 (1992).
- Groettrup, M. et al. *J. Biol. Chem.* **270**, 23808–23815 (1995).
- Realini, C., Dubiel, W., Pratt, G., Ferrell, K. & Rechsteiner, M. *J. Biol. Chem.* **269**, 20727–20732 (1994).
- Mott, J. D. et al. *J. Biol. Chem.* **269**, 31466–31471 (1994).
- Gray, C. W., Slaughter, C. A. & DeMartino, G. N. *J. molec. Biol.* **236**, 7–15 (1994).
- Honore, B., Leffers, H., Madsen, P. & Celis, J. E. *Eur. J. Biochem.* **218**, 421–430 (1993).
- Ahn, J. Y. et al. *FEBS Lett.* **366**, 37–42 (1995).
- Realini, C., Rogers, S. W. & Rechsteiner, M. *FEBS Lett.* **348**, 109–113 (1994).
- Del Val, M. et al. *J. Virol.* **65**, 3641–3646 (1991).
- Rötzschke, O., Falk, K., Faath, S. & Rammensee, H.-G. *J. exp. Med.* **174**, 1059–1071 (1991).
- Del Val, M., Münch, K., Reddehase, M. J. & Koszinowski, U. H. *Cell* **58**, 305–315 (1989).
- Seelig, A., Boes, B. & Kloetzel, P. M. *Enzyme Protein* **47**, 330–342 (1993).
- Koszinowski, U. H. et al. *J. Virol.* **58**, 59–66 (1986).
- Keil, G. M., Fibi, M. R. & Koszinowski, U. H. *J. Virol.* **54**, 422–428 (1985).
- Groettrup, M., Baron, A., Griffiths, G., Palacios, R. & von Boehmer, H. *EMBO J.* **11**, 2735–2746 (1992).
- Rötzschke, O. et al. *Nature* **348**, 252–254 (1990).

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A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome

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THE *Escherichia coli* RNA degradosome is a multi-enzyme complex that contains the exoribonuclease polynucleotide phosphorylase (PNPase) and the endoribonuclease RNase E^{1,2}. Both enzymes are important in RNA processing and messenger RNA degradation³⁻¹⁴. Here we report that enolase and RhlB are two other major components of the degradosome. Enolase is a glycolytic enzyme with an unknown role in RNA metabolism. RhlB is a member of the DEAD-box family of ATP-dependent RNA helicases, which are found in both prokaryotes and eukaryotes¹⁵⁻²⁶. We show that the degradosome has an ATP-dependent activity that aids the degradation of structured RNA by PNPase. Incubation of the degradosome with affinity-purified antibody against RhlB inhibited the ATP-stimulated RNA degradation. These results suggest that RhlB acts by unwinding RNA structures that impede the processive activity of PNPase. RhlB is thus an important enzyme in mRNA turnover.

In addition to RNase E and PNPase, the degradosome contains two major proteins of relative molecular masses 50,000 and 48,000 (p50 and p48, respectively) which have not previously been char-

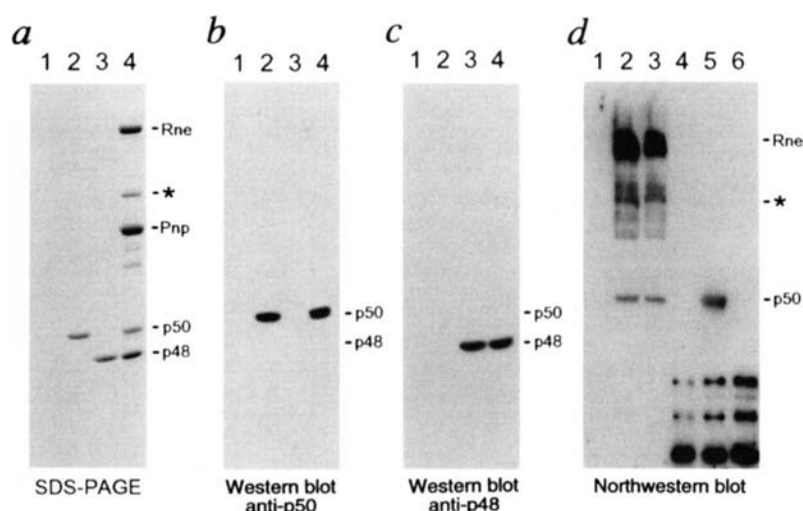
acterized^{1,2}. To identify these proteins, we determined the amino-terminal amino-acid sequence of p50 as SKTHLTEQKFSDFAL, and of p48 as SKIVKIIGREIIDSRL. Based on these data, the proteins were provisionally identified as RhlB²⁶ and enolase²⁷. The genes for RhlB and enolase were cloned, and the corresponding proteins overexpressed in *E. coli* (Fig. 1). The recombinant proteins had the same *M_r* values as the corresponding degradosomal proteins (Fig. 1a), and reacted with affinity-purified antibodies raised against p50 or p48 (Fig. 1b, c), confirming the identities of p50 and p48 as RhlB and enolase, respectively.

DEAD-box proteins such as RhlB typically have ATP-dependent RNA helicase activity^{15,16}. PNPase is an exoribonuclease that degrades RNA processively in the 3' to 5' direction, but this activity is impeded by stem-loop structures in the RNA¹²⁻¹⁴. This suggested that RhlB might act in the degradosome by unwinding such structures to facilitate degradation by PNPase. To test this hypothesis, the activities of two forms of PNPase, in its free state and in the degradosome (that is, with RhlB), were compared in the presence and absence of ATP. A *malE-malF* transcript of 375 nucleotides, which forms a stable RNA stem-loop known to impede degradation by PNPase, was used as a substrate¹²⁻¹⁴. Both forms of PNPase paused at the base of the stem-loop (Fig. 2a, b), generating an intermediate, REP (for repetitive extragenic palindromic)-stabilized RNA (RSR). The addition of ATP promoted degradation of the RSR by the degradosome (Fig. 2a) but not by free PNPase (Fig. 2b). When the RSR intermediate was allowed to accumulate by brief digestion with the degradosome in the absence of ATP, subsequent addition of ATP led to rapid degradation of the RSR (half-life less than 1 min; data not shown). Degradation of the RSR was not promoted by addition of the non-hydrolysable analogue ATP- γ S. Analysis of degradosome-mediated digestion under conditions in which RNase E was inactive showed that ATP still promoted digestion of the RSR (Fig. 2c). Thus the ATP-dependent degradation of the stem-loop does not require RNase E cleavage of the RNA.

When the degradosome was incubated with affinity-purified antibodies against RhlB, the addition of ATP no longer promoted

FIG. 1 Enolase and RhlB are components of the degradosome. a, An 8% SDS-polyacrylamide gel stained with Coomassie brilliant blue. b, c, Western blots of the gel probed with anti-p50 and anti-p48 antibodies, respectively. In a-c, lanes 1, 2 and 3 contain extracts of cells containing the vector plasmid pET11a, or plasmids expressing RhlB or enolase, respectively; lane 4 contains a degradosome preparation. We estimate by densitometric analysis of Coomassie-blue-stained SDS-polyacrylamide gels that the degradosome preparation is 16% RNase E, 28% PNPase, 11% p50 (RhlB) and 18% p48 (enolase) by mass. d, Northwestern blot probed with radiolabelled *malE-malF* RNA. Lane 1, free PNPase; lane 2, degradosome with thermolabile RNase E; lane 3, degradosome with wild-type RNase E; lanes 4-6, lysates of *E. coli* expressing recombinant enolase, RhlB or the pET11a control, respectively. In a and d, the protein (marked by an asterisk) migrating slower than PNPase is a proteolytic product of RNase E^{1,2}.

METHODS. The degradosome was prepared from *E. coli* strain AC21 (wild-type *rne*) or AC22 (temperature-sensitive *rne-3071*) by a modification of a published procedure¹. After ammonium sulphate precipitation, the protein complex was purified by chromatography on SP-Sepharose (Pharmacia) followed by preparative glycerol gradient sedimentation. The *eno* and *rhlB* coding sequences were amplified by the polymerase chain reaction (PCR) from *E. coli* genomic DNA using *Pfu* DNA polymerase (Stratagene), and cloned into the expression vector pET11a (Novagen). The resulting plasmids, containing *eno* and *rhlB* under the control of the bacteriophage T7 *gene10* promoter and ribosome binding site, were transformed into *E. coli* BL21(DE3). Expression was induced with isopropyl- β -D-thiogalactoside (IPTG). Cells were lysed by incubation with lysozyme-EDTA, followed by



freeze-thawing and sonication. After DNase I treatment, extracts were clarified by centrifugation. SDS-PAGE, western blots and affinity-purification of antibodies have been described^{1,2}. Rabbit antibodies against p48 and p50 were raised against the polypeptides from the degradosome separated by preparative SDS-PAGE. The northwestern blot was performed as described except that the tRNA blocking agent was omitted¹⁰. The blot was probed with radioactive *malE-malF* RNA (500 ng, 6.5×10^6 c.p.m.), prepared as described for Fig. 2.

degradation of RSR, which was undigested even after 40 min incubation (Fig. 3a). In a control experiment using preimmune serum (Fig. 3b), the addition of ATP promoted rapid degradation of the RSR. This provides direct evidence that RhlB promotes mRNA degradation by ATP-dependent unwinding of the RNA structures that impede PNPase. In addition, the RhlB antibodies (but not the preimmune serum) increased PNPase pausing at structured RNA sequences less stable than REP, resulting in the accumulation of two degradation intermediates (marked by an asterisk in Fig. 3a) which are normally only observed transiently at early time points (Figs 2 and 3b). As the RhlB antibody does not crossreact with PNPase or any other component of the degradosome (Fig. 1b), the increased pausing must also be a consequence

of antibody binding to RhlB, presumably through RhlB influencing PNPase activity in the degradosome. DEAD-box helicases, such as RhlB, typically exhibit RNA-dependent ATPase activity^{15,16}. The degradosome was shown to have basal ATPase activity of 65 U mg⁻¹, which was stimulated to 187 U mg⁻¹ by addition of the *malE-malF* RNA (Fig. 4). The values are within the range of activities observed for other DEAD-box proteins (3–660 U mg⁻¹)^{18–23}. Yeast RNA and poly(U) stimulated the ATPase activity to the same extent, but single-stranded or double-stranded DNA had no effect (data not shown). RhlB from the degradosome, and overexpressed recombinant RhlB, were shown to bind RNA by a northwestern blot assay (Fig. 1d). Although the recombinant RhlB bound RNA (Fig. 1d), it did not

FIG. 2 The degradosome has an ATP-dependent activity that facilitates exonucleolytic digestion by PNPase. Digestion of RNA using a, the degradosome; b, free PNPase; or c the degradosome with a thermolabile RNase E; time course of digestion in the absence or presence of ATP. Treatment of full-length (FL) RNA with PNPase results in degradation from the 3' end until the enzyme pauses at the base of the stem-loop structure, generating a 300-nucleotide (nt) intermediate (RSR). The FL RNA is stable for the period of the assay in the absence of added exonuclease (data not shown).

METHODS. The radioactive, 375-nt transcript from the *malE-malF* intergenic region RNA was synthesized as described^{13,14} using the plasmid pCH77 digested with *EcoRI*. Highly purified PNPase (free PNPase) was obtained from C. Portier (Institut de Biologie Physico-chimique, Paris). The RNA (100 ng; 1.3×10^6 c.p.m.) was incubated with 1.6 µg degradosome (Fig. 1) or 0.15 µg free PNPase at 37 °C in a final reaction volume of 30 µl containing (in mM) 20 Tris-HCl, pH 7.5, 1.5 DTT, 1 magnesium acetate, 20 KCl, 10 K₂HPO₄. ATP was added where indicated (1 mM final). Samples (2 µl) were withdrawn before (time 0), and 0.5, 1, 2.5, 4, 6, 8, 10, 20 and 30 min after addition of enzyme. The reaction was stopped by adding 15 µl of a formamide-dye loading mix, and 5 µl was then electrophoresed on a 6% sequencing gel. In c, the degradosome preparation from strain AC22 (temperature-sensitive *me-3071*) was preincubated at 45 °C for 8 min, to inactivate the thermolabile RNase E, and assayed at 45 °C.

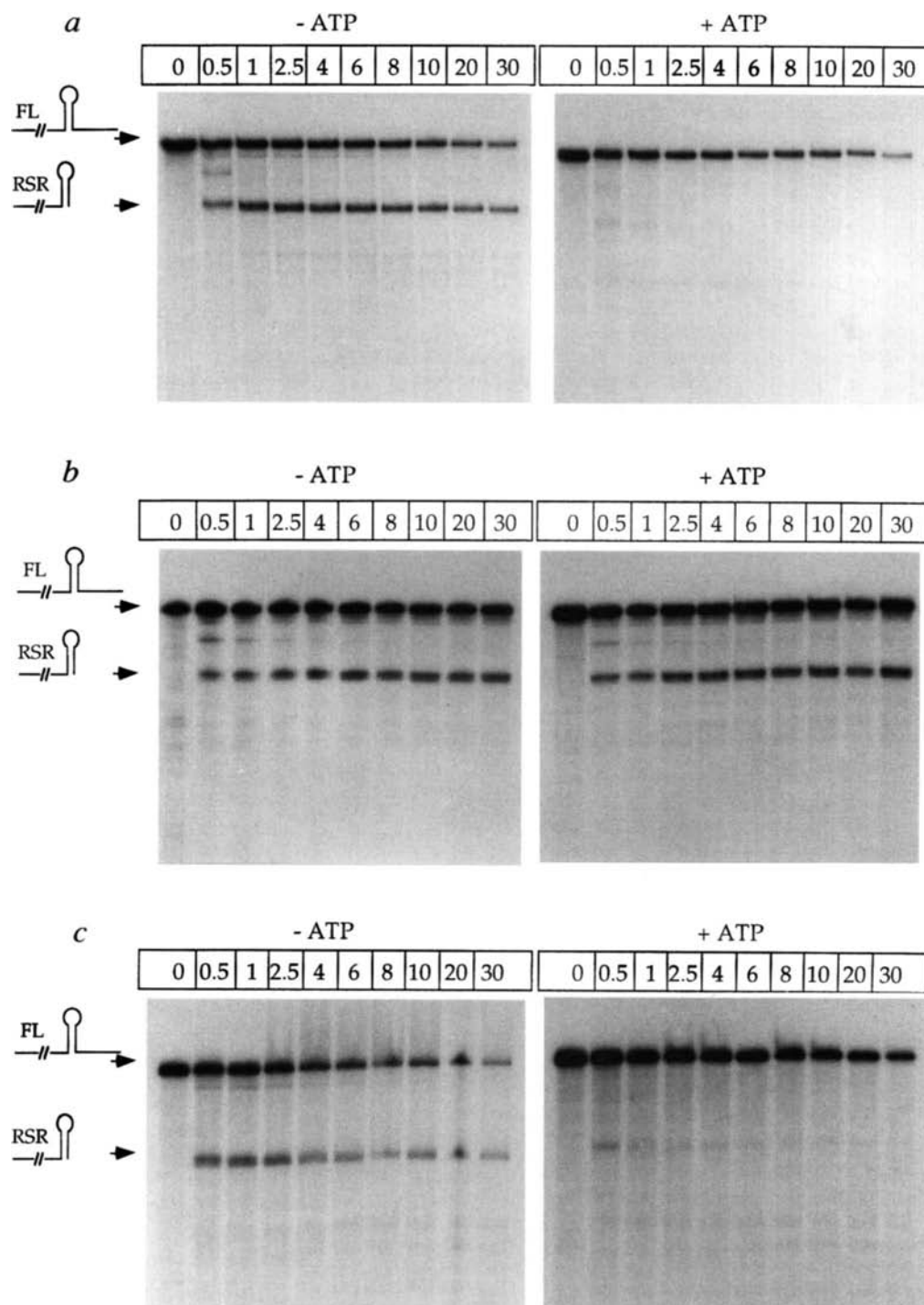


exhibit detectable ATPase activity (data not shown). Thus the enzymatic activity of RhlB appears to require interaction with other components of the degradosome. Another DEAD-box helicase, eIF-4A, needs to interact with additional proteins to exhibit its helicase activity^{24,25}.

The second newly identified component of the degradosome was enolase. The activity of highly purified *E. coli* enolase was previously reported as 147 U mg^{-1} (ref. 27–28). Enolase activity could readily be detected in the degradosome preparation (41 U mg^{-1} ; 18% enolase by mass) and in partly purified extracts of the overexpressed recombinant protein (173 U mg^{-1}). The degradosomal enolase is at least as active as the enzyme purified by classical methods. A northwestern blot assay using either recombinant or degradosomal enolase did not reveal RNA binding (Fig. 1d), and RNA binding could not be detected by ultra-violet crosslinking (data not shown). Thus enolase does not appear to be an RNA-binding protein. An immunoprecipitation experiment showed that only 5–10% of the total enolase in cell extracts is complexed with the degradosome (data not shown). We

previously showed that p48 (enolase) is the β -subunit of PNPase¹, a polypeptide that is not required for catalytic activity but has been found to copurify under some conditions. Enolase is the only component of the degradosome identified thus far that does not have a known function obviously related to RNA turnover.

Our results identify the DEAD-box protein RhlB as a new component of the machinery that mediates mRNA turnover. The advantage of having an RNA helicase as an integral component of the degradosome is self-evident: the unwinding of stem-loops by RhlB would ensure the rapid degradation of RNA by PNPase. These results may be relevant to the finding that 3' polyadenylation can destabilize RNA I, a small, highly structured RNA, by a pathway involving the degradosome²⁹. Poly(A) polymerase adds a 3' tail that converts RNA I to a form more readily degraded *in vivo*, although the mechanism is obscure. Poly(A) polymerase cannot be detected associated with the degradosome (data not shown). However, polyadenylation might facilitate the initiation of degradation by PNPase or activate unfolding of the RNA by RhlB. Whatever the mechanism, it is apparent that the degradosome

FIG. 3 Antibodies against RhlB inhibit the ATP-dependent activity that promotes PNPase digestion of structured RNA. Time courses of RNA degradation by the degradosome following preincubation with affinity-purified anti-p50 (RhlB) antibody (a) or preimmune serum (b), each in the absence or presence of ATP. In a, two transient degradation intermediates (indicated by asterisks) were prominent in addition to the RSR intermediate (marked to the left of a). These transient intermediates are normal intermediates in degradation, but in the absence of antibody pretreatment usually only appear at early time points of digestion (Fig. 2a, b), and correspond to increased degradosome pausing at the base of stem-loop structures¹³.

METHODS. Anti-p50 antibody (Fig. 1) was affinity-purified and concentrated approximately 10-fold, essentially as described². The final concentration of the affinity-purified antibody was less than 0.1 mg ml^{-1} . Purified degradosome ($0.4 \mu\text{g}$) (Fig. 1) was incubated with $10 \mu\text{l}$ of affinity-purified anti-p50 antibody for 90 min at 4°C in a $16\text{-}\mu\text{l}$ reaction. The reaction was then split into two parts: to one half, ATP and radioactive RNA were added; to the other, water and radioactive RNA were added. Samples ($1.5 \mu\text{l}$) were withdrawn at time 0, and 5, 10, 20, 30 and 40 min after the addition of the RNA (164 ng ; $2.8 \times 10^5 \text{ d.p.m.}$). In the control reaction, preimmune serum was diluted to 0.1 mg ml^{-1} in the same buffer used to affinity-purify the anti-p50 antibody. The radioactive RNA substrate, digestion conditions, electrophoresis and autoradiography were as described for Fig. 2.

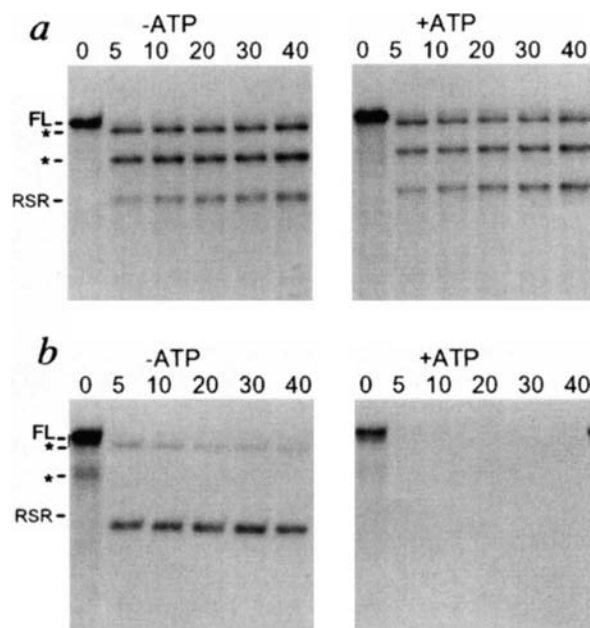
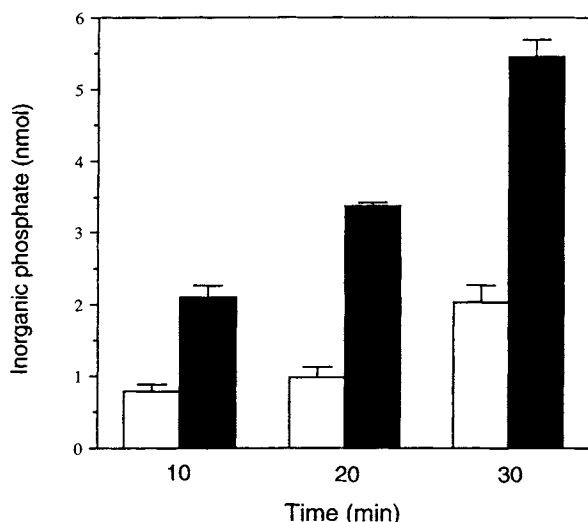


FIG. 4 ATP hydrolysis by the degradosome. ATPase activity was measured by the release of inorganic phosphate (P_i). Assays were performed in the absence (white bars) or presence (black bars) of exogenous RNA. Each bar represents the mean of a triplicate determination, and the error bar shows the standard deviation of the measurement.

METHODS. Inorganic phosphate was measured as described previously²⁹. Purified degradosome ($1 \mu\text{g}$) (Fig. 1) was incubated for 0, 10, 20 or 30 min at 37°C in a final volume of $50 \mu\text{l}$ containing 2 mM ATP. The 375-nt *malE-malF* RNA (non-radioactive; Fig. 2) was added to a final concentration of $4 \mu\text{g ml}^{-1}$. The reaction conditions were as described for the assay of RNase E¹. A trace of inorganic phosphate detected in the 0-min sample (0.21 nmol in the absence or presence of RNA) was subtracted from the 10, 20 and 30 min points before plotting the data. The activity was estimated by taking the average of the rates at each time point (U , $\text{nmol ATP hydrolysed per min}$). The specific activity is expressed per mg of total protein. In the absence of exogenous RNA, the activities were: 10 min, 78 U mg^{-1} ; 20 min, 49 U mg^{-1} ; 30 min, 68 U mg^{-1} ; average, 65 U mg^{-1} . In the presence of exogenous RNA the activities were: 10 min, 210 U mg^{-1} ; 20 min, 169 U mg^{-1} ; 30 min, 181 U mg^{-1} ; average, 187 U mg^{-1} .



containing an RNA helicase can degrade highly structured RNA molecules.

It has been reported that the *rhlB* gene can be deleted without lethal consequences²⁶. However, the putative deletion strain still has a copy of *rhlB*, and more recent attempts to delete the gene failed, indicating that *rhlB* may be essential (our unpublished results; M. Cashel, personal communication). The results reported here provide evidence of a DEAD-box RNA helicase having an active role in bacterial mRNA degradation. □

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1. Carpousis, A. J., Van Houwe, G., Ehretsmann, C. & Krisch, H. M. *Cell* **76**, 889–900 (1994).
2. Py, B., Causton, H., Mudd, E. A. & Higgins, C. F. *Molec. Microbiol.* **14**, 717–729 (1994).
3. Ghora, B. K. & Apirion, D. *Cell* **15**, 1055–1066 (1978).
4. Oro, M. & Kuwano, M. *J. molec. Biol.* **129**, 343–357 (1979).
5. Tomcsányi, L. & Apirion, D. *J. molec. Biol.* **185**, 713–720 (1985).
6. Mudd, E. A., Carpousis, A. J. & Krisch, H. M. *Genes Dev.* **4**, 873–881 (1990).
7. Mudd, E. A., Krisch, H. M. & Higgins, C. F. *Molec. Microbiol.* **4**, 2127–2135 (1990).
8. Lin-Chao, S. & Cohen, S. N. *Cell* **65**, 1233–1242 (1991).
9. Bouvet, P. & Belasco, J. G. *Nature* **360**, 488–491 (1992).
10. Cormack, R. S., Genereaux, J. L. & Mackie, G. A. *Proc. natn. Acad. Sci. U.S.A.* **90**, 9006–9010 (1993).
11. Donovan, W. P. & Kushner, S. R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 120–124 (1986).
12. Newbury, S. F., Smith, N. H., & Higgins, C. F. *Cell* **51**, 1131–1143 (1987).
13. McLaren, R. S., Newbury, S. F., Dance, G. S. C., Causton, H. C. & Higgins, C. F. *J. molec. Biol.* **221**, 81–95 (1991).

14. Causton, H., Py, B., McLaren, R. S. & Higgins, C. F. *Molec. Microbiol.* **14**, 731–741 (1994).
15. Schmid, S. R. & Linder, P. *Molec. Microbiol.* **6**, 283–291 (1992).
16. Fuller-Pace, F. V. *Trends Cell Biol.* **4**, 271–274 (1994).
17. Iost, I. & Dreyfus, M. *Nature* **372**, 193–196 (1994).
18. Grifo, J. A., Abramson, R. D., Satler, C. A. & Merrick, W. C. *J. biol. Chem.* **259**, 8648–8654 (1984).
19. Nishi, K., Morel-Deville, F., Hershey, J. W. B., Leighton, T. & Schnier, J. *Nature* **336**, 496–498 (1988).
20. Hirling, H., Scheffner, M., Restle, T. & Stahl, H. *Nature* **339**, 562–564 (1989).
21. Schwer, B. & Guthrie, C. *Nature* **349**, 494–499 (1991).
22. Fuller-Pace, F. V., Nicol, S. M., Reid, A. D. & Lane, D. P. *EMBO J.* **12**, 3619–3626 (1993).
23. Liang, L., Diehl-Jones, W. & Lasko, P. *Development* **120**, 1201–1211 (1994).
24. Rozen, F. et al. *Molec. cell. Biol.* **10**, 1134–1144 (1990).
25. Pause, A. & Sonenberg, N. *EMBO J.* **11**, 2643–2654 (1992).
26. Kalman, M., Murphy, H. & Cashel, M. *New Biol.* **3**, 886–895 (1991).
27. Klein, M. & Freundl, R. EMBL data bank, accession number X82400 (1994).
28. Spring, T. G. & Wold, F. *Meth. Enzym.* **62**, 323–329 (1975).
29. Xu, F. & Cohen, S. N. *Nature* **374**, 180–183 (1995).
30. Chifflet, S., Torriglia, A., Chiesa, R. & Tolosa, S. *Analyt. Biochem.* **168**, 1–4 (1988).

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CORRESPONDENCE and requests for materials should be addressed to C.F.H. (e-mail: higgins@icrf.icnet.uk).

CORRECTIONS

A canine distemper virus epidemic in Serengeti lions (*Panthera leo*)

Melody E. Roelke-Parker, Linda Munson, Craig Packer, Richard Kock, Sarah Cleaveland, Margaret Carpenter, Stephen J. O'Brien, Andreas Pospischil, Regina Hofmann-Lehmann, Hans Lutz, George L. M. Mwamengele, M. N. Mgasa, G. A. Machange, Brian A. Summers & Max J. G. Appel

Nature **379**, 441–445 (1996).

In this Letter, we neglected to refer to the paper by T. C. Harder *et al.* (*Vaccine* **13**, 521–523; 1995) which reported canine distemper virus P gene sequences from two Serengeti lions that were victims of the same epidemic as those that were the subject of our Letter. M.J.G.A. and M.E.R.-P. were coauthors of the Harder *et al.* paper, which was published in April 1995, before our paper was submitted to *Nature*. The sequences described in that paper were deposited in GenBank with the accession number Z46431. The sequences are identical in both papers, although they were derived from different lions and in separate laboratories. We regret that we omitted to cite this earlier work. □

The structure of the *Escherichia coli* EF-Tu·EF-Ts complex at 2.5 Å resolution

Takemasa Kawashima, Carmen Berthet-Colominas, Michael Wulff, Stephen Cusack & Reuben Leberman

Nature **379**, 511–518 (1996)

In Fig. 5 of this Letter, Asn329 should be Gln329 (panel *b*), Asp348 should be Glu348 (panel *c*), and His73 should be His78 (*f*). Also, the last citation of ref. 10 in the text should be ref. 11. □

A comprehensive genetic map of the mouse genome

William F. Dietrich, Joyce Miller, Robert Steen, Mark A. Merchant, Deborah Damron-Boles, Zeeshan Husain, Robert Dredge, Mark J. Daly, Kimberly A. Ingalls, Tara J. O'Connor, Cheryl A. Evans, Margaret M. DeAngelis, David M. Levinson, Leonid Kruglyak, Nathan Goodman, Neal G. Copeland, Nancy A. Jenkins, Trevor L. Hawkins, Lincoln Stein, David C. Page & Eric S. Lander

Nature **380**, 149–152 (1996)

OWING to a typographical error, the legend to Table 2 reads “For the autosomes, all of the Z-scores are significant at the $P = 0.05$ level . . .”. It should read “For the autosomes, none of the Z-scores is significant at the $P = 0.05$ level . . .”. □

ERRATUM

Modulation of Ca^{2+} channels by G-protein $\beta\gamma$ subunits

Stefan Herlitze, David E. Garcia, Ken Mackie, Bertil Hille, Todd Scheuer & William A. Catterall

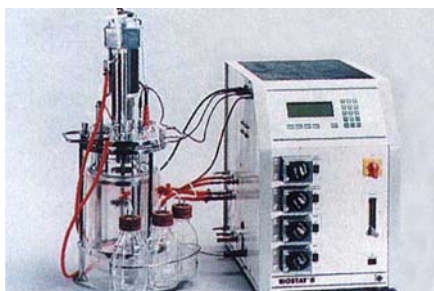
Nature **380**, 258–262 (1996).

FOR the top panel of Fig. 1*d*, GTP- γ S is also included in the intracellular solution as stated in the figure legend, and not GDP- γ S as indicated on the figure itself. □

Microbiology marketplace

This week's issue reviews new technology and laboratory items in the field of microbiology, including a new anaerobe identification system, new gel electrophoresis boxes and a clinical laboratory microscope.

B. BRAUN BIOTECH now offers the Biostat B autoclavable laboratory fermenter system, with 2-, 5-, or 10-litre culture vessels, for small-scale fermentation applications. It has been adapted to the demands of modern biotechnology with flexible process data acquisition, data handling and process control. It can be operated batch-wise and continuously. Moreover, it is equipped with a measurement and control system for pH, pO_2 , antifoam, level and substrate. The culture vessel is mounted on a supporting frame, which also carries a holder for reagent storage



B. Braun Biostat B fermenter.

bottles. This design is said to provide an easy-to-assemble culture vessel and comfortable handling. The digital measurement and control system is designed to ensure safe and user-friendly control of the system, as its functions are specially adapted to the requirements of automated fermenter operation.

Reader Enquiry No. 100

Bibby Sterilin has launched a range of **swab rinse kits**, which may prove to be a valuable tool for routine monitoring and hygiene control. Suitable for sampling any surface, whether wet or dry, the kits should be useful for reaching inside pipe-work, equipment and machinery. The kits should also be useful for sampling surfaces and equipment as part of routine practice in situations where compliance with bio-burden control is essential, such as hospitals, pharmaceutical companies and food manufacturing sites. To satisfy the requirements of different sampling protocols, several swab rinse kits are offered. Each kit comprises a 'peel pouch', containing a single-use swab and a tube of rinse solution. With a choice of large or standard rayon-tipped or alginate swabs, microbiologists can select the most appropriate kit for the task. Accurate total viable counts



Swab rinse kits for routine monitoring.

can be performed using an alginate swab, which dissolves in the rinse solution, releasing all of the bacteria taken up by the swab. A 100 cm² area can be defined using the disposable flexible template. A general-purpose rinse medium for the maintenance of bacteria or fungi also neutralizes disinfectants and sanitizing agents.

Reader Enquiry No. 101

Microbank, designed and developed by Pro-Lab Diagnostics, comprises a **cryovial system** that incorporates treated carrier beads and a cryopreservative solution. This cryogenic preservative solution maintains the integrity of bacterial cells during prolonged periods of frozen storage. This product should prove useful for



Cryovial cryopreservative beads.

the routine quality control needs of microbiologists. It offers economical and convenient performance with a wide range of bacteria. Full references and data are available upon request. Applications of the cryovial system include research collections, reference collections, teaching, quality control and resistance studies.

Reader Enquiry No. 102

Becton Dickinson Microbiology Systems has announced the availability of the new BBL Crystal **anaerobe identification system**. The new system identifies clinically-significant anaerobes in only four hours, without requiring a specialized anaerobic chamber or any reagent additions. It employs modified conventional, fluorogenic and colorimetric substrates to identify frequently isolated anaerobic bacteria from clinical specimens. The system is said to offer standardized quality control and cost-efficient ease of use. There are three separate media-specific databases in both electronic and manual formats: Schaedler blood agar, CDC anaerobe blood agar and alternate blood agars.

Reader Enquiry No. 103

Electrophoresis

By incorporating a smoke-grey stage into its new **electrophoresis gel boxes**, Molecular Bio-Products hopes to make gel loading easier than ever. The stage makes sample wells clearly distinguishable, and should help eliminate loading errors. This electrophoresis gel box utilizes a gel-casting tray and comb system designed for straight, even wells. The gel-casting tray transmits ultraviolet, so that gels do not have to be handled during gel analysis. The procedure involves removing the tray from the gel box, carrying it to the dark room and photographing the gel. If the gel needs to run longer, it can be returned to the gel box by setting the tray back in the gel box. This feature should prove useful for working with delicate, low-melting point agarose.

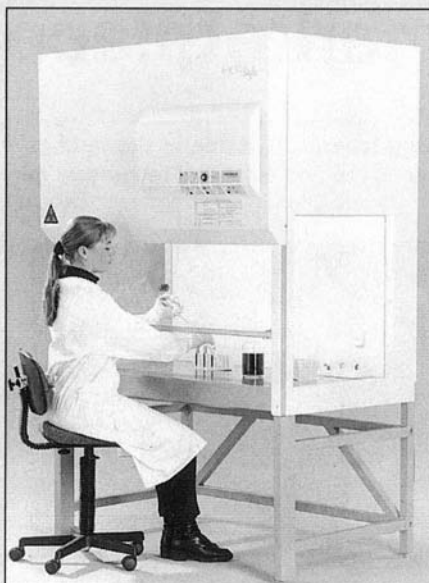
Reader Enquiry No. 104

The **microgel horizontal electrophoresis system** from Owl Scientific is now supplied as a complete system, including all components required to cast and run a gel. Gels are cast onto 5-cm × 7.5-cm glass slides inside the casting chamber and then a seven-well comb is placed into the slot of the chamber to form wells. The running chamber requires only 100 ml of buffer and gels can be run in minutes. A lid and power cords are also supplied with the system. Owl also offers a complete line of chemical reagents and power supplies to meet all electrophoresis needs.

Reader Enquiry No. 105

The Herasafe **microbiological safety cabinet** from Heraeus Instruments is designed to provide users with maximum safety. This cabinet is now equipped with a negative pressure area, so that should a leak occur, it is dealt with safely. The cabinet is said to be easy to disinfect — when closed, the motor-driven sliding window completely seals off the work chamber, permitting safe disinfection with formaldehyde, for example, without the need for sealing tape. All maintenance work can be done from the front of the unit and a new clamping device makes it easier to change the filters. The unit has an all-steel design, front and side windows are made of non-reflecting safety glass and a work space that is uniformly and brightly lit. Other features include quiet operation, segmented stainless steel shelves that can be autoclaved and protected electrical outlets and media connectors.

Reader Enquiry No. 106



Microscopy

The new Leica DM LS **clinical laboratory microscope** features include Delta infinity optics for high-quality imaging and a new method of heat compensation to prevent partial warming of the microscope. The microscope has an ergonomic design with various 'ergomodels'. These allow for individual adjustment, such as viewing height, providing comfortable, fatigue-free working conditions at the microscope. The curved shape of the Ernest Igl design is said to ensure high stability and optimal layout of all controls.

Reader Enquiry No. 107

Leo has announced the release of the LEO 430, 440 and 435VP **scanning electron microscopes**, which now include as standard a newly designed specimen stage featuring complete motorization of all five axes. These models incorporate ergonomic dual-joystick control for precise and easy specimen manipulation and an LCD display indicating axis positions. The new stage is compatible with all existing Leo stage software options. Full magnification-compensated motion is designed to make allowance for fast specimen survey at all magnifications. Tilt compensation is also included. Stage-coordinate storage software is an extra option, which is designed to aid the inspection of large uneven samples.

Reader Enquiry No. 108

Laboratory equipment

For laboratories where automated equipment is a luxury, the Stat-Matic **bottle-top dispenser** from TriContinent with optimal

eight-port manifold offers the user a convenient, affordable solution. Unlike multi-channel pipettors, which must be refilled after each single row is dispensed, the Stat-Matic plate washer/filler can be attached to a large-capacity reservoir with 20-, 24- or 28-mm threaded adapters. Researchers can continually dispense volumes of 100 µl, 200 µl or 250 µl until the fluid in the reservoir has been depleted. Volume selection is internal and can be changed easily by the operator. An eight-port manifold, designed for exact placement into the microplate wells, delivers fluids with a stated row-to-row accuracy of five per cent.

Reader Enquiry No. 109

Jouan has announced the availability of a brochure describing their KR422 **high-capacity, floor-model centrifuge**. This centrifuge is capable of centrifuging up to 6.4 litres and can achieve a relative centrifugal force of 7,300g. Standard features include microprocessor control, refrigeration, a four-rotor system and charts that are designed to make accessory selection easy for the various applications. This model features a control panel that is angled for improved visibility, a lid that opens automatically, a flat easy-to-clean screen, clear messages and a host of safety features, which include a steel guard barrier ring, an imbalance detector and dual-lid interlock. The P60 high-capacity blood-bank rotor allows 12 triple bags with satellites to be processed per run. Special buckets are available to accommodate blood-bag packs with leukocyte trap filters. Aerosol containment systems are available for safe centrifugation of toxic and pathogenic samples.

Reader Enquiry No. 110

Catalogues

Sigma Chemical has released its new 1996 **CD-ROM catalogue**, with a extensive, built-in index for fast, full-text searching under Folio Views electronic publishing software. This edition includes a 'shopping cart' function to facilitate ordering, as well as full-colour photographs of equipment and supplies. The CD-ROM catalogue is compatible with DOS, Windows and Macintosh computers.

Reader Enquiry No. 111

Fisher Scientific is now offering its 1996 **life science catalogue**, which brings together a comprehensive range of products from various manufacturers. This catalogue has been expanded to over 300



Fisher Scientific's life science catalogue.

pages and now offers products in various disciplines, including electrophoresis, microbiology, molecular biology, biochemistry and cell biology.

Reader Enquiry No. 112

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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